Métaux traces : réponses écophysiologiques et rôle dans le maintien du polymorphisme de coloration mélanique du plumage chez le pigeon biset

Marion Chatelain

Université Pierre et Marie Curie

Ecole doctorale Science de la Nature et de l'Homme

Institut d'Ecologie et des Sciences de l'Environnement de Paris

/ Equipe écophysiologie évolutive

Métaux traces : réponses écophysiologiques et rôle dans le maintien du polymorphisme de coloration mélanique du plumage chez le pigeon biset

Par Marion Chatelain

Thèse de doctorat d'Ecologie

Dirigée par Julien Gasparini et Adrien Frantz

Présentée et soutenue publiquement le 30 septembre 2015

Devant un jury composé de :

Mme. Martine Maïbèche	Professeur, Université Pierre & Marie Curie, Paris (France)	Présidente
M. Marcel Eens	Professeur, Université d'Anvers, Anvers (Belgique)	Rapporteur
M. Pierre Bize	Directeur de recherche, Université d'Aberdeen, Aberdeen (Ecosse)	Rapporteur
M. Thomas Lenormand	Directeur de recherche, CEFE/CNRS, Montpellier (France)	Examinateur
M. Julien Gasparini	Maître de conférences, Université Pierre & Marie Curie, Paris (France)	Directeur de thèse
M. Adrien Frantz	Maître de conférences, Université Pierre & Marie Curie, Paris (France)	Directeur de thèse

Remerciements

Je ne suis pas très douée pour dire merci alors j'espère que vous serez indulgents.

Adrien et **Julien,** évidemment je vous remercie d'avoir eu confiance en moi et de m'avoir laissé les rênes de votre projet. Même si je l'ai quelques fois regretté, je vous remercie de m'avoir laissé une quasi-totale liberté dans mes recherches. D'ailleurs vous l'avez peut-être aussi regretté en voyant la facture… Je vous remercie également pour ce que vous êtes, deux jeunes chercheurs dynamiques avec qui il est agréable de travailler et de discuter de science, mais surtout deux charlots avec qui il est plus qu'agréable de discuter des sujets les plus diverses (que parfois l'on a oublié, ou que l'on préfèrerait oublier…). Merci pour votre humour qui fait du bien au moral et allège ces longues journées de travail. Malgré ce que certains peuvent dire, j'espère ne pas avoir usé avec vous d'un ton un peu trop autoritaire et ne pas vous avoir traumatisé à vous « rappeler à l'ordre », disons, assez fréquemment...

Marcel Eens, Pierre Bize, je vous remercie d'avoir accepté d'évaluer mais surtout de participer à l'amélioration de mon travail de thèse. Merci également à **Thomas Lenormand** en tant qu'examinateur, et à **Martine Maïbèche,** en tant que présidente de mon jury de thèse d'avoir accepté de participer à la discussion autour de mes recherches.

Alexandre et **Renaud,** je vous remercie pour ces discussions très enrichissantes que nous avons eues lors de mes comités de thèse. Merci pour vos conseils et vos idées.

Je remercie également toutes les personnes avec qui j'ai collaboré : **Emmanuel Aubry, Florence Le Cornec** et **Irina Djouraev**, pour leur aide dans la réalisation des dosages des métaux traces, **Frédéric Angelier** pour la réalisation des dosages hormonaux, **Sarah Laclaire** pour la réalisation des analyses bactériennes et d'odeur et **Kazumasa Wakamatsu** pour la réalisation des dosages de mélanine.

Je tiens à remercier toutes les personnes de **l'équipe écophysiologie évolutive**. **Claudy**, je te remercie tout particulièrement pour l'aide précieuse que tu m'as apporté dans la réalisation des analyses de laboratoire, ta patience et ton intérêt pour mettre en place de nouveaux protocoles, mais aussi pour ta participation à l'ambiance quelque fois agitée de notre bureau.

Merci également à **tous les autres membres du laboratoire** pour leur sympathie et les discussions à la cafet'. Merci **Pierre** pour ton aide sur le sexage, en bricolage, ainsi que de nous permettre de faire les mots croisés, un moment incontournable de nos pauses déjeuner.

Merci **Béatriz, Samuel** et toute **l'équipe du CEREEP** pour les coups de main que vous m'avez donnés sur le terrain. Foljuif a été pour moi un véritable havre de paix durant la belle saison et un vrai calvaire durant l'hiver. Je me rappellerai toujours de ces quelques nuits effrayantes que j'ai passées seule au chalet.

Thibault, Salomé, Bérangère, Sébastien, Janice, Nicole, Caroline, Anaïs, Fabien, Elodie, Sakina, je vous remercie pour votre aide sur le terrain et en laboratoire et pour votre compagnie souvent très sympathique. L'encadrement de stagiaires a été pour moi très riche en enseignements. Plusieurs d'entre vous sont maintenant en thèse, et même si je n'y suis pas pour grand-chose, cela me fait plaisir d'avoir contribué à votre intérêt pour la science, ou tout du moins de ne pas vous en avoir dégoûté.

Sarah, je suis très contente que l'on ait pu partager cette saison de terrain ensemble. Merci pour l'aide que tu m'as apporté, ainsi que pour tes idées. J'espère que notre collaboration sera fructueuse et pourquoi pas que l'on travail de nouveau ensemble par la suite !

Merci à mes collègues doctorants, ou plutôt devrais-je dire à mes amis : **Floriane, Thomas, Clémentine, Ewen, Marie, Anaïs**. On ne peut jamais savoir comment se seraient passées les choses si on avait changé ceci ou cela, mais je ne pense pas prendre trop de risque en disant que sans vous, cette grande aventure aurait été beaucoup moins fun. **Floriane,** que de soirées déprimes nous avons passées à nous dire que l'on ferait mieux d'ouvrir notre restaurant de tartes (« Les deux quiches »), mais on se serre les coudes depuis la licence et je ne saurais rêver meilleure amie. **Thomas,** comment sans toi ne me serais-je pas emmêlée dans des boucles ! Merci à toi pour l'ambiance parfois bon enfant, parfois déchaînée, parfois houleuse, parfois orageuse, ou parfois encore dansante de notre bureau.

Ma famille, merci d'avoir fait semblant de vous êtes intéressé aux recherches que j'ai menées durant ma thèse bien que je suspecte que vous n'en voyiez pas vraiment l'intérêt. **Yves,** merci surtout à toi pour avoir cru en moi quand mon moral était au plus bas, merci pour ton optimisme inébranlable.

Enfin, merci encore à **tout le monde**. Même si ces trois années ont parfois été difficiles et que j'avais une petite voix dans la tête qui me suggérait de tout plaquer, je ne regrette rien et je n'ai rien perdu de ma motivation à poursuivre ma carrière dans la recherche. Cela a été un réel plaisir que de travailler dans ce laboratoire, et bien que je souhaite découvrir d'autres horizons, j'ai peur de regretter l'ambiance qui y régnait.

Sommaire

Introduction à l'écologie urbaine

La population humaine ne cesse de s'accroître et est accompagnée par la densification et l'expansion des villes. En 2000, les villes recouvraient 0,5% de la superficie mondiale des terres émergées et cet espace devrait tripler d'ici 2030 (Seto et al., 2012). L'urbanisation est associée à de profondes modifications de l'environnement d'origine. Ainsi, l'environnement urbain est entre autres caractérisé par une forte densité de population humaine, un taux de recouvrement par des infrastructures élevé, une fragmentation des habitats, une pollution chimique, lumineuse, sonore, électromagnétique et thermique importante, etc. Evidemment, ces modifications des facteurs abiotiques de l'environnement sont susceptibles d'affecter la structure et le fonctionnement de l'ensemble de l'écosystème. L'écologie urbaine s'intéresse aux interactions entre les êtres vivants et l'environnement biotique et abiotique qui les entoure, au niveau des écosystèmes urbains. Ainsi, les études en écologie urbaine ont permis de mettre en évidence une tendance à la diminution de la richesse spécifique avec le degré d'urbanisation de l'habitat (McKinney, 2008). Ce milieu semble notamment sélectionner des espèces généralistes et opportunistes, c'est-à-dire ayant peu d'exigences au niveau de leur régime alimentaire ou de leur d'habitat (e.g. le pigeon biset est une espèce dite commensale, dans le sens où, bien qu'à l'origine granivore, elle se nourrisse des déchets laissés par l'Homme). Par ailleurs, des différences notables sont mesurées entre les individus des populations urbaines et rurales, comme un avancement de la phénologie des plantes (Neil and Wu, 2006), un avancement de la période de reproduction de certaines espèces d'oiseaux (Partecke et al., 2004), des différences de coloration du plumage (Eeva et al., 2008), etc. Alors que la plus part des études comparent les populations d'une même espèce en fonction du degré d'urbanisation de leur habitat, peu d'entre elles permettent d'identifier les facteurs environnementaux responsables des différences mesurées. Or l'identification de ces facteurs apparaît essentielle à la mise en place de plans de conservation et la réduction de l'impact écologique des activités humaines, un des buts fondamental de l'écologie urbaine appliquée.

Les métaux traces dans l'environnement urbain

Les métaux traces, autrement appelés éléments-traces métalliques (EMT) ou à tort métaux lourds (i.e. certains métaux traces ont une masse atomique faible ; e.g. l'aluminium), sont des métaux ou métalloïdes qui, comme leur nom l'indique, sont présents en faibles quantités dans l'environnement ($<1 g.kg^{-1}$ dans les sols). Cette appellation générique sous-entend également la notion de toxicité pour l'Homme, bien que certains soient des oligoéléments essentiels (e.g. zinc, cuivre, fer). Du fait de l'absence de cadre officiel autour de la définition de métal trace, la liste des éléments définis comme tels est arbitraire. Néanmoins, les métaux retenus par les instances françaises sont l'aluminium, l'arsenic, le cadmium, le chrome, le cuivre, l'étain, le fer, le manganèse, le mercure, le nickel, le plomb, le sélénium et le zinc (arrêté ministériel du 2 février 1998, Académie des Sciences).

Les métaux traces sont naturellement présents dans l'environnement et peuvent être émis dans l'atmosphère par des processus naturels. Par exemple, des émissions de mercure, de plomb et de cadmium au niveau des pôles ont été attribuées à des activités bactériennes (Pongratz and Heumann, 1999). De plus, des métaux sont rejetés dans l'atmosphère via les gaz et les poussières volcaniques ou encore les embruns marins (Bertine and Goldberg, 1971; Nriagu, 1989). Néanmoins, le relargage des métaux traces dans l'atmosphère et leur accumulation subséquente dans les eaux et les sols résulte principalement d'activités humaines (Nriagu, 1979). En effet, de nombreux métaux ont été et sont parfois toujours utilisés en industries et en agriculture pour leurs propriétés physico-chimiques et biologiques. A titre d'exemple, le plomb a fait l'objet d'une utilisation massive lors de la révolution industrielle en Europe et aux Etats-Unis (entre 1850 et 1940), en tant qu'additif antidétonant dans les carburants, agent anticorrosion dans les peintures, conducteur dans les batteries, ou encore stabilisateur de pigments dans les teintures textiles. Le caractère polluant des métaux traces est attribuable au fait qu'ils ne soient pas (bio)-dégradables. Aussi, et malgré quelques mesures pour réduire l'émissions de certains d'entre eux (interdiction de l'ajout de plomb dans les carburants en 1975 et en 2000 aux Etats-Unis et en France respectivement), les métaux s'accumulent dans les sols.

Les émissions de métaux traces étant majoritairement d'origines anthropiques, les concentrations environnementales de ces éléments traces dépendent du degré d'urbanisation du milieu. Le fer, le manganèse, le zinc et le plomb sont les métaux traces généralement les plus abondants en milieux urbains (Tableau 1 ; Azimi et al., 2005; Biasioli et al., 2006; Bilos et al., 2001; Chen et al., 1997; Maas et al., 2010; Manta et al., 2002) mais le plomb est l'élément dont la différence de concentration mesurée entre le milieu urbain et le milieu rural est la plus grande. Ainsi, des

études reportent par exemple des taux de déposition atmosphérique du plomb trois fois plus élevés en milieu urbain (Créteil, France) qu'en milieu rural (St Brisson, Parc naturel régional du Morvan, France ; Azimi et al., 2003), et des concentrations en plomb dans le sol quatorze fois supérieures dans les villes de Washington (Columbia, Etats-Unis) et Baltimore (Maryland, Etats-Unis), que dans les régions rurales environnantes (Roux and Marra, 2007).

City	Hg	Pb	Zn	Cu	Cd	Cr	Co	Ni	V	Sb	Mn	Reference
Rome		330.8			0.31							Angelone et al. (1995)
Pittsburg	0.51	398			1.2							Carey et al. (1980)
Boston		800										Spittler and Feder (1979)
Warsaw		57	166	31	0.73	32	5.1	12			337	Czarnowska (1980)
Hamburg		218.2	516	146.6	2.0	95.4		62.5			750	Lux (1986)
Salamanca		53.1			0.53							Sánchez-Camazano et al. (1994)
Coruña		309	206	60	0.3	39	11	28		3		Cal-Prieto et al. (2001)
Central Madrid		621										Pellicer (1985)
Madrid		161	210	71.7		74.7	6.42	14.1	30		437	De Miguel et al. (1998)
Bangkok		47.8	118	41.7	0.29	26.4		24.8			340	Wilcke et al. (1998)
Aberdeen		94.4	58.4	27		23.9	6.4	14.9			286	Paterson et al. (1996)
Birmingham		570										Department of the Environment (1982)
Glasgow		216	207	97	0.53							Gibson and Farmer (1986)
Central London		647										Rundle and Duggan (1980)
Greater London		250										Rundle and Duggan (1980)
Outer London		322										Davies et al. (1979)
London boroughs		294	183	49	1.0							Culbard et al. (1988)
London		294	183	73	1.0							Thornton (1991)
Hong Kong		93.4	168	24.8	2.18							Li et al. (2001)
Hong Kong		100	93.9	27.5	1.89							Wong et al. (1996)
Hong Kong		89.9	58.8	16.1	0.94							Chen et al. (1997)
Manila		213.6	440	98.7	0.57	114		20.9			1999	Pfeiffer et al. (1988)

Tableau 1. Concentrations en métaux traces (mg.kg-1) mesurées dans les sols de villes Européennes (Manta et al., 2002).

Réponses écophysiologiques aux métaux traces

L'inquiétude particulière face à la pollution aux métaux traces résulte en premier lieu de la biodisponibilité des métaux sous forme ionique. En effet, les métaux sont bioassimilables par les organismes vivants, par voie orale (i.e. absorption des métaux présents dans l'eau et la nourriture), aérienne (i.e. absorption des métaux présents dans l'atmosphère lors de la respiration) ou, chez les plantes, par voix racinaire (i.e. absorption des métaux présents dans l'eau du sol). Leur taux d'absorption (i.e. la proportion de métaux à laquelle un organisme est exposé passant les barrières de l'organisme) dépend fortement de la voie d'exposition, du métal en question, de l'espèce, de l'âge des individus ou encore de leur exposition à d'autres éléments chimiques (e.g. chez l'Homme, le taux d'absorption du cadmium est de 10 à 50% lorsqu'il est inhalé, contre 6% lorsqu'il est ingéré ; ce taux monte à 9% chez les personnes carencées en fer). Une fois absorbés, les métaux sont difficilement éliminés et sont alors stockés dans tout ou partie de l'organisme où ils s'accumulent tout au long de la vie de l'organisme (e.g. chez les vertébrés, le cadmium s'accumule dans les organes filtreurs que sont les reins et le foie).

Comme pour la majorité des polluants (e.g. polluants organiques persistants), les individus des niveaux trophiques supérieures (i.e. carnivores et piscivores) sont ceux montrant des concentrations en métaux traces les plus élevées. La bioamplification des métaux via leur transfert à travers la chaîne alimentaire est responsable de ce qui est appelé la bioconcentration, c'est-à-dire des concentrations en métaux traces dans les organismes supérieures à celles mesurées dans leur environnement (Figure 1). Néanmoins, il existe une corrélation positive forte entre les concentrations environnementales et les concentrations biologiques. De ce fait, les individus habitant en milieu urbain présentent des concentrations biologiques en métaux supérieures à celles des organismes de milieu rural (Roux and Marra, 2007; Scheifler et al., 2006).

Biodisponibilité : Disponibilité d'éléments nutritifs ou toxiques présents dans l'environnement (sol, eau, air) pour les organismes vivants (plantes, champignons, animaux). Elle conduit à la bioassimilation.

Bioaccumulation : Capacité de certains êtres vivants à absorber et stocker des éléments chimiques, dans tout ou une partie de leur organisme. Ces éléments peuvent être essentiels (oligoéléments) ou non (éléments toxiques). Elle est le résultat d'une absorption et d'une rétention de ces éléments supérieure à leur élimination. A titre d'exemple, les champignons sont des organismes bioaccumulateurs de métaux traces (Michelot et al., 1998).

Bioamplification : Aussi appelée biomagnification, c'est le processus par lequel les concentrations de certains éléments chimiques augmentent à chaque niveau de la chaîne trophique (Figure 2).

Bioconcentration : Processus par lequel les concentrations de certains éléments chimiques sont plus élevées dans tout ou une partie de l'organisme que dans son habitat. Elle est souvent la conséquence de la bioamplification.

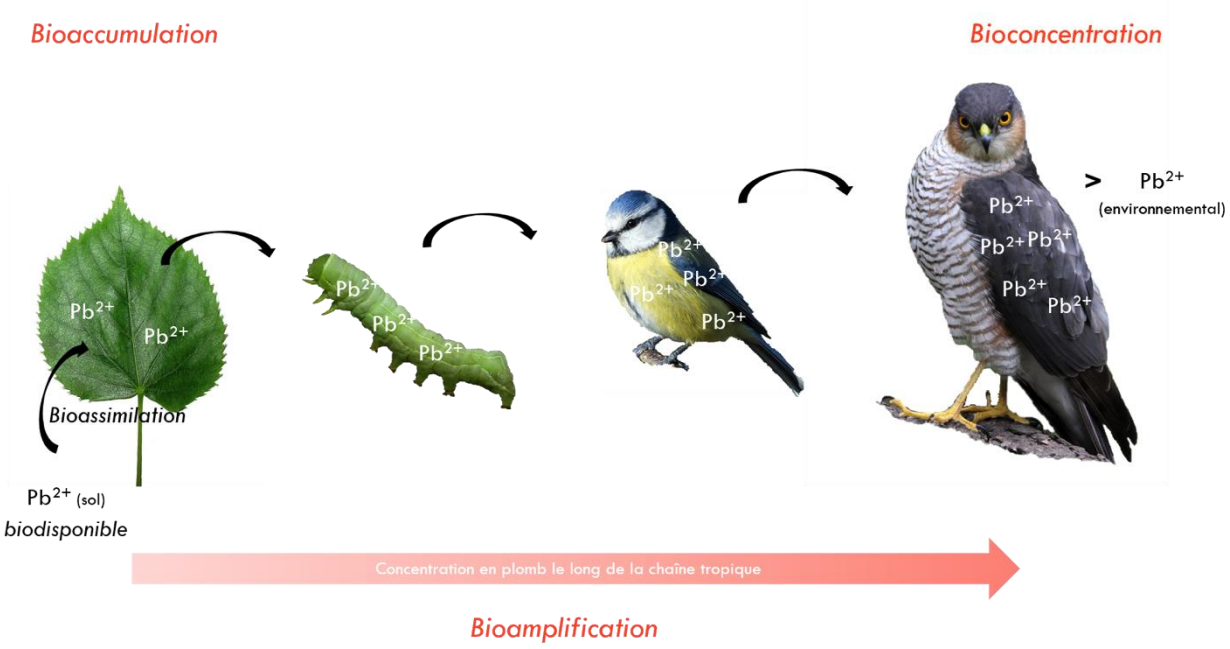


Figure 1. Schéma d'illustration des concepts de biodisponibilité, bioassimilation, bioaccumulation, bioconcentration et bioamplification à travers un exemple sur les concentrations en plomb chez plusieurs individus de niveaux trophiques différents. (Images de Krzysztof Jasiutowicz, Philippe Mothiron, Sébastien Chenal, J. Fouarge).

Une fois absorbés, les métaux traces ne sont pas des éléments inertes. D'après leur définition même, ce sont des éléments traces toxiques. En effet, les métaux traces perdent facilement des électrons ; ils se retrouvent de ce fait sous forme de cations (ions positivement chargés), alors très réactifs. La toxicité des ions métalliques résulte de deux mécanismes principaux :

D'une part, les ions métalliques peuvent avoir une affinité forte avec les sites de fixation de certaines protéines, impliquées par exemple dans la croissance cellulaire, l'apoptose, la réparation de l'ADN, etc., et ainsi désactiver ces protéines. Par exemple, le plomb (Pb^{2+}) est capable de se substituer aux ions bivalents comme le zinc (Zn^{2+}) et le calcium (Ca^{2+} ; Godwin, 2001). Or le zinc et le calcium sont deux oligoéléments essentiels, le premier étant notamment impliqué dans la synthèse de l'hème et le second étant nécessaire à la croissance osseuse et à la libération des neurotransmetteurs. Aussi, le plomb est entre autres responsable d'anémies (Schwartz et al., 1990) et de troubles neurologiques (Bressler et al., 1999; Marchetti, 2003; Toscano and Guilarte, 2005).

D'autre part, la majorité des métaux traces induisent des dommages oxydatifs (Nuran Ercal et al., 2001). Les métaux traces peuvent être divisés en deux catégories : les métaux dits « redox-actifs », dont font partie le fer et le cuivre, et les métaux dits « redox-inactifs », qui comprennent le plomb, le cadmium, le nickel ou encore le mercure. Les premiers catalysent des réactions d'oxydation (e.g. réaction de Fenton) aboutissant à la formation de dérivés réactifs de l'oxygène, aussi appelées espèces réactives oxygénées, dont les principales sont le radical hydroxyle ($\bullet OH$), le peroxyde d'hydrogène (H_2O_2) et l'oxygène singulet ($O_2^{\bullet -}$); la présence d'électrons en valence rend ces radicaux libres très réactifs. Les métaux « redox-inactifs », entrainent quant à eux la déplétion des antioxydants composés de groupes thiols sur lesquels les métaux se fixent (e.g. glutathion) ; la fixation des métaux sur ces protéines les rendes inactives (Koivula and Eeva, 2010).
Néanmoins, il advient de préciser que certains de ces éléments métalliques sont des oligoéléments essentiels à de nombreuses réactions métaboliques. C'est notamment le cas du zinc, du fer et du cuivre. Par exemple, le zinc est connu pour son rôle dans la mise en place d'une réponse immunitaire mais aussi dans la diminution du stress oxydatif ; il est pour ces raisons utilisé dans des thérapies ou en complément alimentaire préventif contre des troubles de l'immunité (Prasad, 2009). Cependant, le zinc peut par exemple avoir des effets neurotoxiques en cas d'exposition à des concentrations élevées (Greenberg and Briemberg, 2004). Aussi la toxicité des métaux traces est dose-dépendante.

Les effets biochimiques des métaux traces se répercutent à l'échelle de l'individu et aux niveaux supérieurs (population, communauté). Néanmoins, à l'origine, les métaux ont été jugés comme toxiques du fait des effets nocifs qu'ils engendrent chez l'Homme et, bien que ces effets puissent être généralisés à d'autres espèces, la majorité des études réalisées dans l'optique d'identifier les effets des métaux portent sur l'être humain. Ces études ont été initiées en réponse à l'apparition de maladies graves, comme le saturnisme, chez les ouvriers d'usines métallurgiques et chez les enfants des familles habitant proches de ces usines. Ainsi, chez l'Homme, les métaux traces sont responsables de diverses maladies pouvant entrainer ou non le décès des individus. Par exemple, le cadmium a été désigné comme responsable de maladies rénales et cardiovasculaires, ainsi que de cancers ; le plomb a quant à lui un fort effet neurotoxique et est responsable de troubles neurologiques affectant notamment les capacités d'apprentissage et de mémorisation, ainsi que l'équilibre psychique des individus (Jarup, 2003). Toujours pour des raisons de santé publique, et parce que les sols agricoles ont été pollués via l'épandage de boues d'épuration, l'écoulement d'eaux usées et l'utilisation de fertilisants, les effets des métaux traces ont été étudiés sur les plantes de cultures ; les métaux peuvent engendrer des cas de chlorose (production insuffisante de chlorophylle), des dégâts oxydatifs, des retards de croissance et de la sénescence (Yadav, 2010). Dans ce même contexte agricultural, des effets toxiques des métaux traces ont été observés sur les microorganismes du sol (Giller et al., 1998).

Alors que de nombreuses études toxicologiques ont permis de définir les doses létales des métaux sur des animaux de laboratoire, les études testant les effets des métaux traces sur les espèces sauvages sont quant à elles plus rares. Chez les oiseaux, il a été montré que les métaux traces affectaient négativement l'immunité (chez la mésange charbonnière et le diamant mandarin: Snoeijs et al., 2005, 2004) et la capacité d'apprentissage (chez le goéland argenté: Burger and Gochfeld, 2004). Par ailleurs, plusieurs études corrélatives menées chez des passereaux montrent une corrélation négative entre le niveau d'exposition aux métaux traces et le succès reproducteur (ie. fort taux de désertion du nid, d'échec à l'éclosion et de mortalité des juvéniles aux sites où les concentrations environnementales en métaux sont les plus élevées ; Eeva et al., 2009; Eeva and Lehikoinen, 1996; Sens et al., 2003), ainsi qu'un lien positif entre le niveau d'exposition aux métaux traces et les dommages oxydatifs (Berglund et al., 2007).
Néanmoins, les études corrélatives menées ne permettent pas de tester les effets des métaux traces de façon exclusive, puisqu'il est possible que d'autres paramètres environnementaux varient conjointement aux concentrations environnementales en métaux traces (e.g. le degré de pollution lumineuse ou sonore). De plus, en milieu naturel, les individus sont exposés à un assemblage de métaux, et ces études ne permettent pas de dissocier les effets de chacun des

métaux pris séparément. Par ailleurs, les études expérimentales réalisées jusqu'à présent sont rares, mesurent un unique trait physiologique (e.g. immunité) et testent l'effet d'un unique métal dont les concentrations sont souvent plus élevées à celles que peuvent rencontrer les individus dans leur milieu naturel. Finalement, alors que les métaux traces sont majoritairement émis par les activités humaines et se retrouvent par conséquent particulièrement concentrés en villes, peu d'études portent sur les effets des métaux traces sur les organismes urbains. Bien que les métaux traces, retrouvés à des concentrations élevées en milieux urbains, soient susceptibles d'affecter significativement la survie et le succès reproducteur des individus, et ainsi d'affecter le fonctionnement, la dynamique et l'évolution des populations, peu d'études permettent d'évaluer la réelle menace que représentent les métaux traces sur la biodiversité urbaine.

LEXIQUE

Immunité :	Mécanismes biologiques de défense d'un organisme contre des pathogènes extérieurs.
Stress oxydatif :	Déséquilibre entre la production de radicaux libres et la capacité de l'organisme à les éliminer (e.g. production d'antioxydants comme le glutathion) ou à réparer les dommages qu'ils génèrent.

Rôle des métaux traces dans le maintien du polymorphisme de coloration mélanique du plumage

En milieu naturel, les organismes sont exposés à un assemblage de métaux, susceptibles d'avoir des effets synergiques ou antagonistes. En effet, alors que l'exposition à certains métaux traces, comme le plomb, est associée à des échecs de la reproduction, une réponse immunitaire moindre, des problèmes neurologiques, etc., d'autres métaux, comme le zinc, ont des effets positifs sur l'immunité ou le stress oxydatif. Les effets physiologiques engendrés par les métaux sont susceptibles d'affecter la survie et le succès reproducteur à long terme des individus, c'est-à-dire leur aptitude à transmettre leurs gènes. Bien que les métaux, à un niveau d'exposition donné, aient des effets soit négatifs, soit positifs, les réponses physiologiques des individus d'une même population sont variables ; autrement dit, le degré de sensibilité aux métaux est variable au sein d'une population. Lorsque le résultat de l'addition des effets engendrés par les métaux traces, qui est fonction de la proportion des différents métaux dans l'environnement, est négatif, les individus les moins sensibles aux métaux traces, c'est-à-dire ceux dont les réponses physiologiques sont les moins affectées lorsqu'exposés aux métaux traces, devraient avoir une espérance de vie et un succès reproducteur supérieur aux autres individus de la population. Ainsi, les phénotypes associés à une meilleure aptitude phénotypique en milieu pollué en métaux traces devraient être sélectionnés. La toxicité des métaux traces étant majoritairement due à leurs effets oxydants et au fait qu'ils se substituent à d'autres éléments essentiels comme le zinc ou le calcium, des phénotypes associés à une faible absorbance des métaux, une forte capacité de détoxication (i.e. d'excrétion ou de fixation des métaux) ou une capacité antioxydante élevée seraient avantagés.

La mélanine est le pigment le plus répandu dans le règne animal ; elle est responsable de la coloration brune à noire (eumélanine) et rousse (phéomélanine) des téguments et notamment des phanères (i.e. plumage, fourrure, écailles). De nombreuses espèces animales présentent un polymorphisme au niveau de la coloration mélanique de leurs phanères (e.g. moineau domestique, mésange charbonnière, écureuil gris, vipère aspic, phalène du bouleau, etc). Le mélanisme est essentiellement sous contrôle génétique (Roulin, 2004, mais voir Bókony et al., 2008; Evans et al., 2000). Pa exemple, chez le pigeon biset, la coloration mélanique du plumage montre un taux élevé d'héritabilité (h^2=0,82 ± 0,12 ; Jacquin et al., 2013; Figure 2).

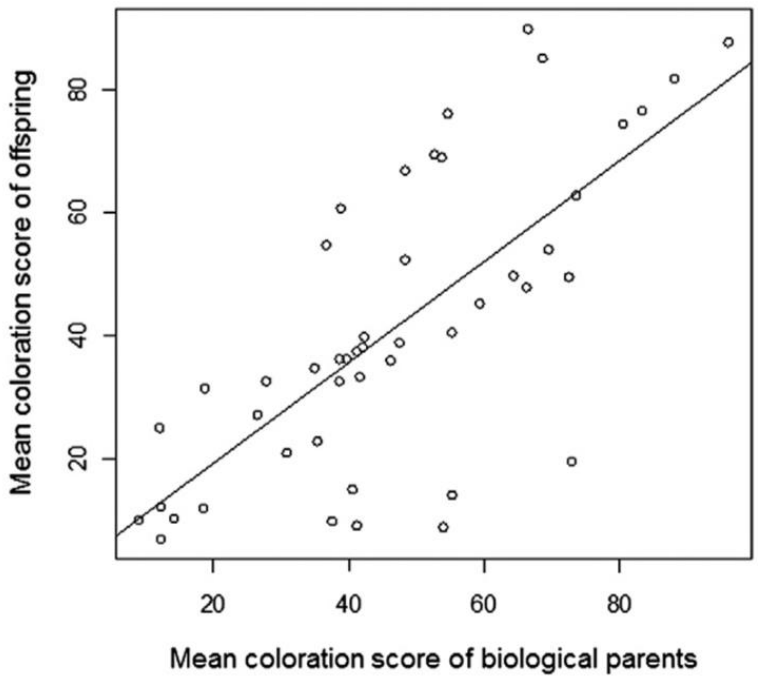


Figure 2. La coloration du plumage moyenne d'une fratrie est positivement corrélée à la coloration du plumage moyenne de leurs deux parents (régression linéaire, $F_{1,45}$=48,57, P<0,001, r^2=0 ,51 ; Jacquin et al., 2013)

Notons néanmoins que quelques études suggèrent que, chez certaines espèces au moins, le mélanisme du plumage soit androgènes-dépendant (Bókony et al., 2008; Evans et al., 2000). Par exemple, les moineaux domestiques supplémentés en testostérone présentent un badge mélanique plus large que les individus non supplémentés (Evans et al., 2000). Ainsi, le mélanisme du plumage constituerait un signal honnête de la compétitivité des mâles (Bókony et al., 2008).

Des études *in vitro* ont montré que la mélanine est capable de chélater certains cations métalliques au niveau de ses groupements carboxyles, hydroxyles et amines négativement chargés (Bridelli and Crippa, 2007; Larsson and Tjälve, 1978; Liu et al., 2004). Peu d'études ont testé la relation entre les concentrations en métaux traces et celles en mélanine *in vivo*. Une relation positive a été mesurée entre la concentration en zinc et le niveau de phéomélanine chez la chouette effraie (Niecke et al., 1999). Par ailleurs, chez le faucon crécerelle, la partie distale noire des plumes contient davantage de plomb que la partie proximale plus claire (Dmowski et al., 1984), alors qu'aucune différence de concentration en plomb n'a été mesurée entre les plumes blanches et noires chez le bec-en-ciseaux noir, la sterne pierregarin et la sterne fulgineuse (Gochfeld et al., 1991). Néanmoins, l'accumulation des métaux dépend de leurs concentrations environnementales au moment de la formation de la plume, susceptibles de varier au cours de la mue. De plus, elle dépend de la distance des barbules par rapport à la base de la plume (i.e. l'accumulation de plomb est plus forte dans les parties distales de la plume chez le faucon crécerelle ; Dmowski et al., 1984). Aussi, il est difficile de tester le lien entre la

coloration mélanique et la concentration en métaux dans le plumage dans ces deux dernières études, qui respectivement comparent différentes plumes d'un même individu ou différentes parties d'une même plume, d'autant plus que la mue n'a pas été synchronisée.
Bien qu'aucune étude ne démontre de façon tangible une relation positive entre la concentration en mélanine d'un tégument et sa capacité à chélater des ions métalliques, l'effet chélateur de la mélanine est suggéré comme un des rôles principal de la mélanine (Hong and Simon, 2007; McGraw, 2003).

D'une part, la chélation des ions métallique serait un moyen efficace de détoxication des métaux. Plus particulièrement, chez les oiseaux, le transfert des métaux traces dans les plumes lors de la mue via la mélanine permettrait de diminuer les concentrations sanguines en métaux et leur accumulation dans les organes internes. La plume, une fois sa croissance terminée, est dévascularisée, c'est-à-dire qu'elle n'est plus reliée à la circulation sanguine. Aussi, les métaux se retrouvent piégés dans la plume et sont éliminés complètement lors de la mue suivante. Par conséquence, nous faisons l'hypothèse que les individus au plumage le plus mélanique (i.e. le plus noir ou roux) sont capables de transférer davantage de métaux traces potentiellement toxiques dans leurs plumes et ainsi possèdent une plus grande capacité de détoxication. Ces individus seraient alors moins sensibles à une exposition chronique aux métaux traces par rapport aux individus plus clairs. Néanmoins, certains métaux traces sont des oligoéléments essentiels à faibles concentrations ; c'est par exemple le cas du zinc, du fer et du cuivre. Il est généralement admis que leurs concentrations sanguines sont strictement régulées (Evans et al., 1973); ceci suggère que seul l'excédent serait transféré dans les plumes. Dans le cas où cette régulation homéostatique ne serait pas avérée (i.e. le transfert ne dépendrait pas des concentrations sanguines), le transfert des métaux traces essentiels dans les plumes pourrait être responsable de carences et serait en cela délétère pour les individus. De ce fait, nous pouvons penser que les bénéfices potentiels associés au transfert des métaux traces dans les plumes dépendent des concentrations relatives des métaux traces bénéfiques versus toxiques dans l'environnement ; par exemple, dans un environnement riche en zinc et en plomb, le transfert de ces métaux dans le plumage serait avantageux, alors que dans un environnement pauvre en zinc et en plomb, ce transfert serait désavantageux puisqu'il conduirait à séquestrer des oligoéléments dans des parties inertes du corps. Dans le cas particulier d'un environnement pauvre en zinc mais riche en plomb, l'avantage associé au transfert des métaux dans les plumes dépendrait d'un compromis entre le maintien de concentrations sanguines en zinc optimales et la détoxication du plomb. Ce compromis est susceptible de varier, selon si les métaux occupent les mêmes sites de fixation à la mélanine (Hong and Simon, 2007; Liu et al., 2004) et selon leur affinité pour ces sites (Hong and Simon, 2007; Larsson and Tjälve, 1978).

D'autre part, la production de la mélanine est contrôlée par un gène responsable de la synthèse de la pro-hormone POMC (pro-opiomélanocortine) dont la modification génère quatre mélanocortines (ACTH, α-MSH, β-MSH, γ-MSH) se fixant sur le récepteur à la mélanocortine MC1R (Figure 3). Or, les mélanocortines se fixent à quatre autres récepteurs (MC2R, MC3R, MC4R et MC5R) impliqués dans de nombreux processus physiologiques comme la réponse au stress, l'activité anti-inflammatoire, cardiovasculaire et sexuelle, etc. De ce fait, la synthèse de la mélanine est corrélée à de nombreux traits biologiques (Figure 3 ; Ducrest et al., 2008). Par exemple, les pigeons bisets au plumage le plus eumélanique présentent une intensité en endoparasites sanguins plus faible et une réponse inflammatoire plus forte que les pigeons les plus clairs (Jacquin et al., 2011). De plus, le degré d'eumélanisme du plumage chez la chouette effraie est positivement corrélé à la résistance au stress oxydatif (Roulin et al., 2011) et au stress physiologique (Almasi et al., 2012, 2010). Par conséquent, les effets pléiotropes liés à la synthèse de la mélanine peuvent conférer un avantage aux individus au plumage le plus mélanique, plus à même de résister aux effets oxydants et immunosuppresseurs des métaux traces.

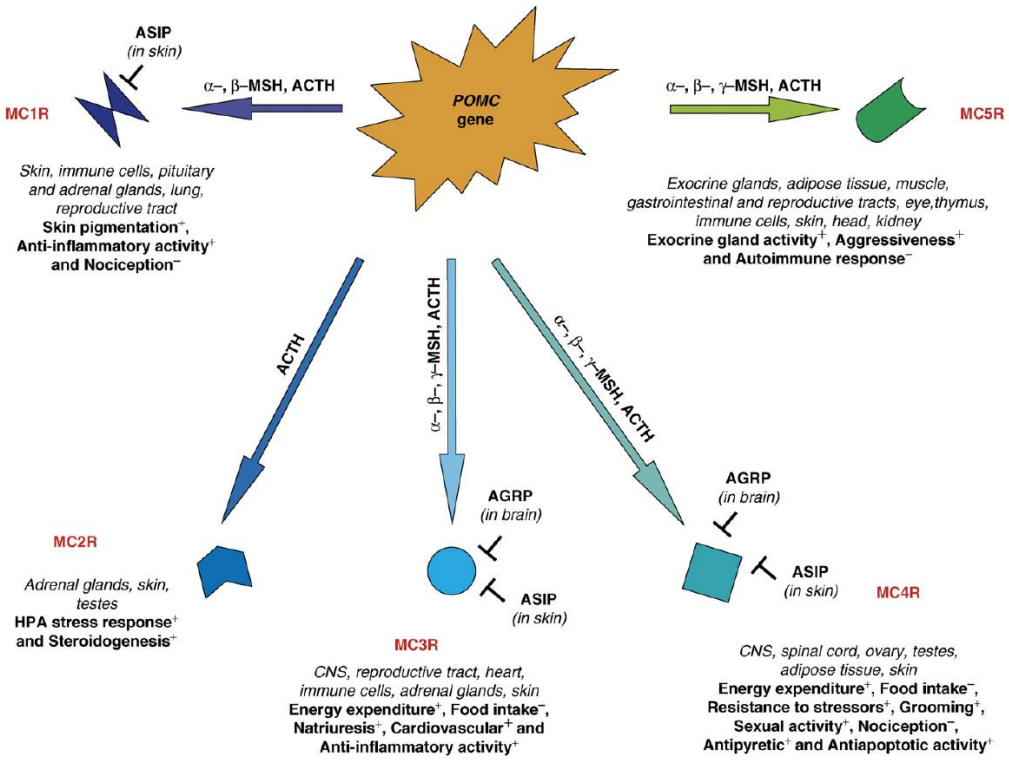


Figure 3. Pléiotropie du gène POMC : la modification de la pro-hormone POMC (pro-opiomélanocortine) génère cinq mélanocortines (ACTH, α-MSH, β-MSH) qui se fixent sur cinq récepteurs (MC1R, MC2R, MC3R, MC4R et MC5R), impliqués dans de nombreux processus physiologiques dont la synthèse de mélanine (Ducrest et al., 2008).

Chez les espèces présentant un mélanisme du plumage fortement héritable, les effets directs (chélation des ions métalliques) et indirects (résistance au stress oxydatif, aux parasites et au stress physiologique) de la synthèse de mélanine sont susceptibles de sélectionner les oiseaux au plumage le plus mélanique dans les milieux exposant les individus à de fortes concentrations en métaux traces toxiques. En accord avec cette hypothèse, des études précédentes ont mis en évidence de plus fortes fréquences de pigeons bisets foncés (i.e. davantage eumélaniques) dans les villes, où les concentrations en métaux traces sont les plus fortes (Azimi et al., 2003; Kekkonen et al., 2012; Maas et al., 2010; Roux and Marra, 2007; Scheifler et al., 2006), qu'en milieu rural (Jacquin et al., 2013; Obukhova, 2007; Figure 4). De la même façon, une autre étude a montré une corrélation négative entre la taille du badge mélanique chez la mésange charbonnière et la distance par rapport à une usine métallurgique (Dauwe and Eens, 2008). Néanmoins, à ma connaissance, aucune étude n'a cherché à tester l'avantage sélectif à avoir un plumage davantage mélanique dans les milieux pollués en métaux traces.

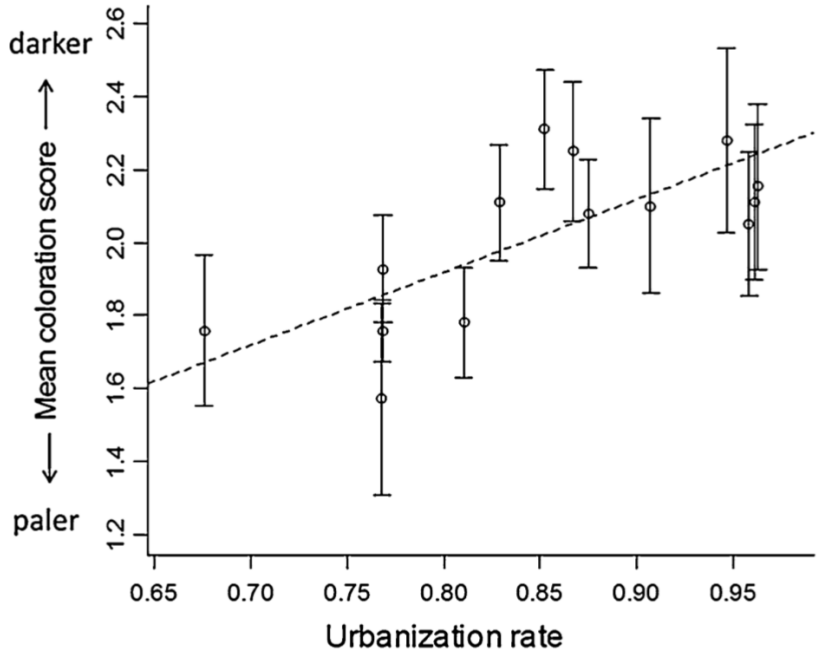


Figure 4. La coloration du plumage moyenne dans une population est positivement corrélée au degré d'urbanisation de l'habitat de cette population (Jacquin et al., 2013).

Du fait des effets pléiotropes liés à la synthèse de mélanine, la mélanisation du plumage, outre l'avantage potentiel qu'elle conférerait face à une exposition chronique à des métaux traces toxiques, est susceptible de refléter différents traits biologiques des individus comme leur capacité à résister aux parasites, leur statut social ou encore leur capacité antioxydante. Le polymorphisme de coloration mélanique observé chez de nombreuses espèces peut résulter de quatre mécanismes évolutifs : l'avantage de l'hétérozygotie, la sélection fréquence-dépendante, le polymorphisme transitoire, ou l'adaptation à des niches écologiques différentes. Cette dernière hypothèse suggère des bénéfices et des coûts liés au mélanisme qui diffèrent selon l'habitat, du fait de l'hétérogénéité spatiale et temporelle de l'environnement. Ces bénéfices et coûts peuvent être dus à la fois à la quantité de mélanine synthétisée (i.e. degré de mélanisme), mais également au type de mélanine produit (i.e. eumélanine vs. phéomélanine). L'apparition et le maintien de la coloration rousse (phéomélanique) des téguments est une question peu testée et qui pourtant est considéré par certains auteurs comme une aberration évolutive (Hill and Hill, 2000). En effet, la synthèse de phéomélanine requière l'utilisation de cystéine (Benathan et al., 1999, 1992; Ito et al., 2000), également indispensable à la synthèse de glutathion (Williamson et al., 1982), l'un des antioxydants majeurs chez l'ensemble des organismes vivants (Alscher, 1989; Meister, 1992). De plus, la phéomélanine est phototoxique, puisqu' elle entraine la production de radicaux libres lorsqu'elle est exposée à des radiations ultraviolettes (Samokhvalov et al., 2007). Aussi, identifier les bénéfices et les coûts associés à la quantité et le type de mélanine synthétisé est nécessaire à la compréhension du maintien du polymorphisme de coloration mélanique.

LEXIQUE

Chélation :	Processus physico-chimique de liaison entre un ligand (chélateur ; e.g. mélanine) et un cation métallique.
Détoxication :	Processus physico-chimique ou métabolique aboutissant à la désactivation ou à l'excrétion de substances toxiques pour l'organisme.
Pléiotropie :	Qualité des gènes ou des protéines impliqués dans plusieurs caractères phénotypiques apparemment indépendants.
Niche écologique :	Ensemble des conditions biotiques et abiotiques propres à une localisation et un moment donné.

Durant ma thèse, j'ai cherché à évaluer la toxicité des métaux traces sur les populations animales en milieu urbain, et à tester le rôle adaptatif du mélanisme du plumage dans un tel environnement. J'ai conduit une étude semi-corrélative ainsi que deux études expérimentales chez le pigeon biset (*Columba livia*), une espèce urbaine modèle, qui plus est montrant une forte variabilité au niveau de la coloration mélanique de son plumage (Figure 5). Je me suis plus particulièrement intéressée à deux métaux que sont le plomb et le zinc. Alors que le premier est un métal toxique non essentiel (Godwin, 2001), le second est avant tout un oligoélément essentiel (Prasad, 2009, 1998), néanmoins toxique à des concentrations élevées (Greenberg and Briemberg, 2004). Ces deux métaux sont par ailleurs particulièrement abondants dans l'environnement urbain, à la fois dans l'atmosphère, les eaux pluviales et les sols (Azimi et al., 2005; Biasioli et al., 2006; Bilos et al., 2001; Chen et al., 1997; Maas et al., 2010; Manta et al., 2002). Le plomb est notamment le métal montrant les concentrations environnementales les plus contrastées entre les milieux ruraux et urbains (Azimi et al., 2003; Roux and Marra, 2007). Par ailleurs et par conséquent, le zinc est de loin le métal le plus accumulé dans le plumage des pigeons bisets provenant de populations urbaines (Frantz et al., 2012) et le plomb y est lui aussi particulièrement abondant (Frantz et al., 2012). Pour finir, les liens négatifs et positifs entre l'immunité et respectivement les concentrations en plomb et en zinc dans les plumes (Gasparini et al. 2014) soulignent l'importance plus que probable de ces deux métaux sur la faune urbaine.

J'ai tout d'abord testé le rôle de la mélanine dans la fixation des métaux traces au niveau des plumes, c'est-à-dire le lien entre la coloration mélanique du plumage et les concentrations en métaux traces dans les plumes, au cours d'une étude semi-corrélative (Chapitre 1) et une étude expérimentale (Chapitre 2). J'ai ensuite testé le rôle de la mélanine dans la détoxication des métaux traces, c'est à dire le lien entre la coloration mélanique du plumage et les concentrations sanguines en métaux traces (Chapitre 2). Finalement, j'ai testé les effets écotoxicologiques engendrés par une exposition aux métaux traces ainsi que l'effet de la coloration mélanique du plumage dans la modulation de ces effets. Dans un même temps, j'ai comparé les réponses écophysiologiques des individus en fonction de la coloration mélanique de leur plumage (degré de mélanisme et type de mélanine), indépendamment de leur exposition aux métaux traces. Ces trois grandes questions ont été abordées conjointement sur différents paramètres biologiques que sont la reproduction (Chapitre 2), l'immunité (Chapitre 3), les transferts maternels précoces (Article 4) et la communauté bactérienne du plumage (Chapitre 5).

Problématiques

Quelles sont les réponses écophysiologiques d'une exposition chronique aux métaux traces et quelles pressions de sélection exercent-ils sur la coloration mélanique du plumage?

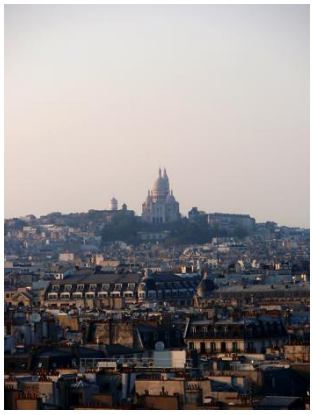
1) Quels sont les effets écotoxicologiques d'une exposition chronique aux métaux traces ?

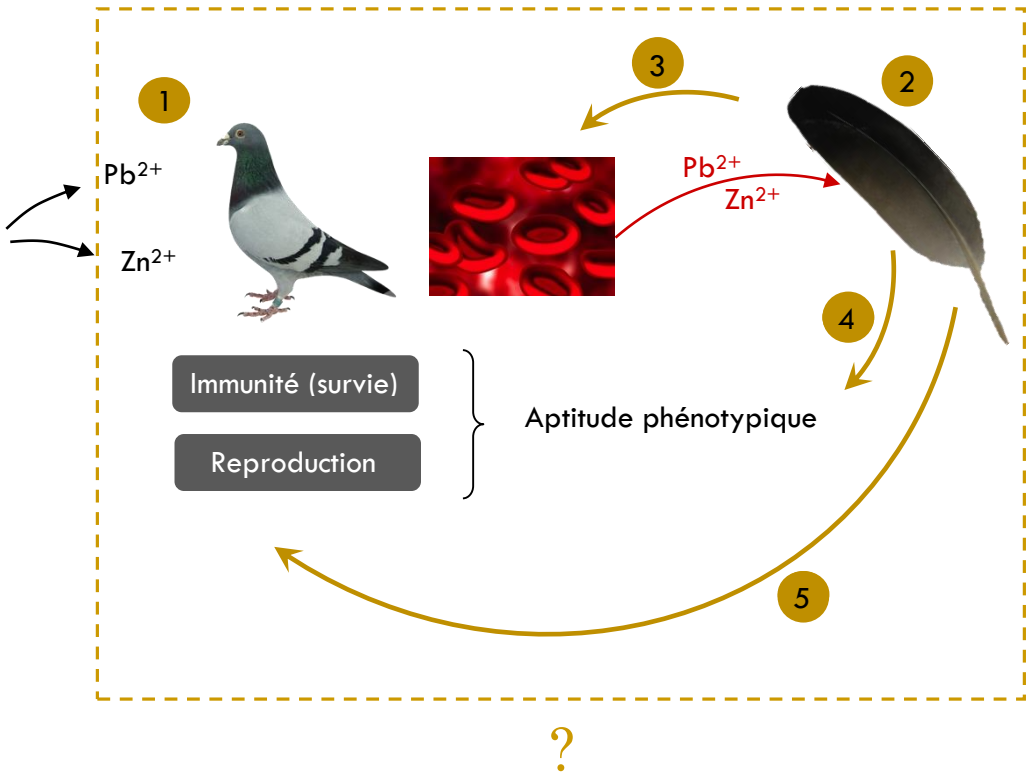
2) Le mélanisme du plumage est-il lié à la capacité à séquestrer les métaux dans les plumes ?

3) Le mélanisme du plumage permet-il la détoxication des métaux traces ?

4) Le mélanisme du plumage module-t-il les effets écotoxicologiques induits par les métaux traces ?

5) Le mélanisme du plumage est-il représentatif de différences physiologiques entre les individus ?





Modèle biologique : le pigeon biset

Origine - Le pigeon biset (*Columba livia*) est une espèce d'oiseau de la famille des *Columbidae*. L'espèce sauvage d'origine aurait évolué d'une part par synanthropie, et d'autre part du fait de la sélection artificielle exercée sur les individus domestiqués (N.B. la domestication du pigeon biset aurait débuté il a 5000 à 10000 ans). De nombreux individus se sont régulièrement échappés des élevages (i.e. phénomène de marronnage) ; la mise en contact secondaire et la reproduction des pigeons bisets sauvages et des sous-espèces et races ayant évolué par domestication et synanthropie serait à l'origine des populations actuelles de pigeons bisets. Aussi, les pigeons bisets actuels, parfois appelés pigeons des villes puisque habitant principalement les milieux urbanisés, présentent des caractères phénotypiques différents de ceux de l'espèce d'origine. En effet, la domestication et la synanthropie seraient à l'origine du polymorphisme observé au niveau de la coloration du plumage (Figure 5), mais aussi de leur période de reproduction allongée et de leur tolérance à la proximité de l'Homme (Johnston and Janiga, 1995).

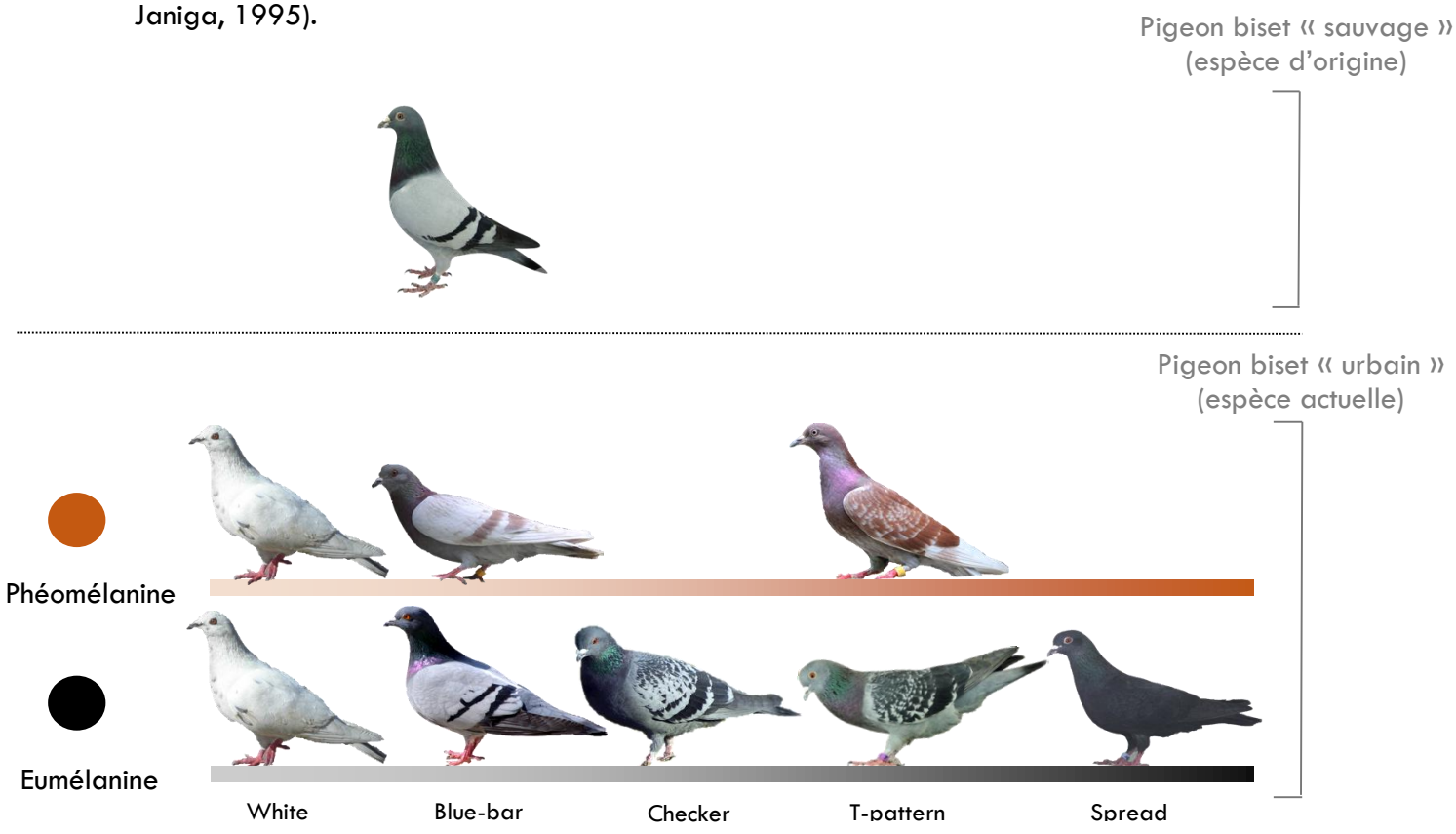


Figure 5. Coloration mélanique (phéomélanique ou eumélanique) du pigeon biset ancestral, dit « sauvage » et de l'espèce descendante actuelle, dite « urbaine » ou « des villes ».

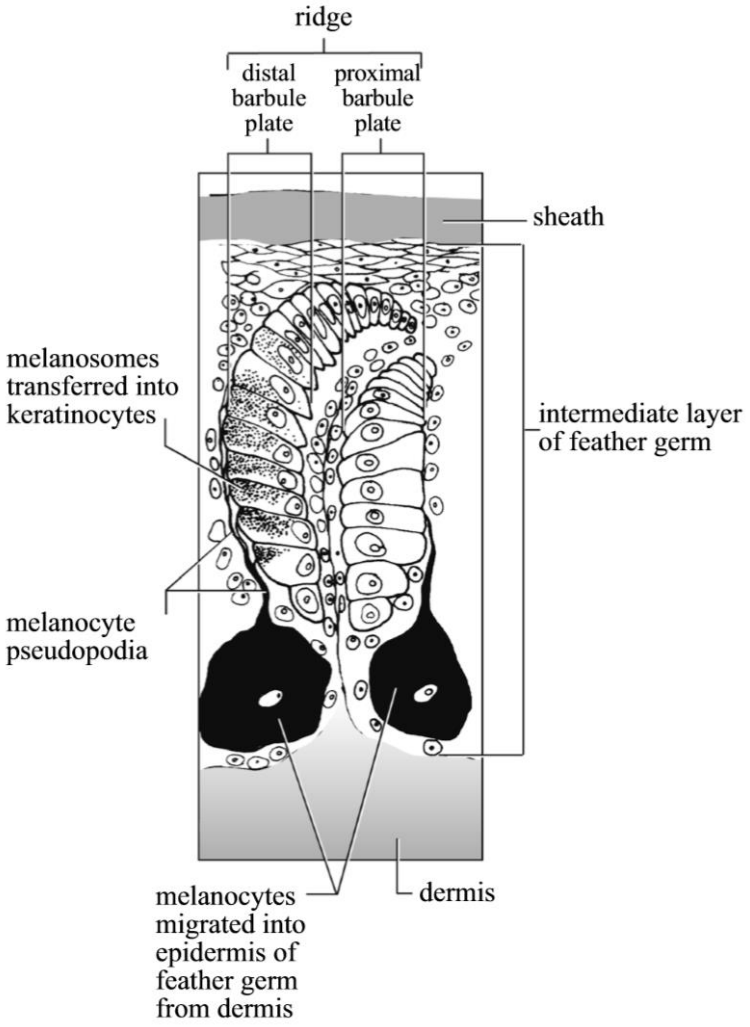
Reproduction – Le pigeon biset est considéré comme monogame et, contrairement à beaucoup d'espèces qui forment un couple stable pour la durée d'une saison de reproduction, le pigeon biset fait partie des rares espèces à former un couple pour la vie. Il a une reproduction quasi continue au cours de l'année, bien que l'essentiel de la reproduction ait lieu d'avril à octobre. Les femelles pondent généralement 2 œufs par ponte et font de 1 à 6 pontes par an. La période d'incubation des œufs varie de 16 à 22 jours. Les juvéniles prennent leur envol environ 30 à 35 jours après éclosion (Figure 6). L'investissement dans les soins aux descendants est biparental, à la fois lors de l'incubation des œufs, la thermorégulation et l'alimentation des jeunes au nid (N.B. mâles et femelles sécrètent dans leur jabot une substance riche en protéines et en lipides, appelée lait de jabot, avec laquelle ils nourrissent les juvéniles en début de croissance) et parfois l'alimentation post-envol des juvéniles (Johnston and Janiga, 1995).

Figure 6. De la ponte à l'envol : croissance des pigeons bisets juvéniles

Mue – Du fait de leur dégradation au cours du temps, par abrasion abiotique ou par leur dégradation par des bactéries, champignons ou acariens, les plumes, essentielles au vol et à la thermorégulation, doivent être renouvelées. Chez le pigeon biset, la totalité du plumage est renouvelée une fois par an. La mue (i.e. la perte suivie de la repousse des plumes) s'étend sur plus de 6 mois et présente un pic de fin août à mi-octobre ; la mue est par conséquent séquentielle et toutes les plumes ne sont pas renouvelées de façon simultanée. Chez les juvéniles, les premières plumes émergent 5 jours après éclosion et la croissance des plumes dites « juvéniles » est complète à l'âge de 30 jours. Une mue post-juvénile à lieu 50 jours après éclosion, durant laquelle le plumage « juvénile » et remplacé par le plumage « adulte » (Johnston and Janiga, 1995).

Les plumes sont des phanères, c'est-à-dire des productions épidermiques fortement kératinisées. Lors de leur croissance, à savoir lorsque les plumes sont irriguées par la circulation sanguine, peut avoir lieu l'incorporation de mélanines dans la kératine (Encadré 1), responsable alors de la pigmentation de ces téguments. Une fois sa croissance terminée, la plume ne reçoit plus aucun apport sanguin ; c'est une structure inerte (morte).

1. La mélanine est synthétisée au niveau des mélanocytes.
2. Les mélanocytes migrent du derme au germe de la plume (aussi appelé papille).
3. Les mélanocytes se prolongent en pseudopodes permettant le transfert des mélanosomes des mélanocytes aux kératinocytes par phagocytose.
4. La phagocytose des mélanosomes entraine la libération de la mélanine dans les kératinocytes.



Encadré 1. Incorporation de la mélanine dans les plumes. D'après Prum and Williamson (2002).

Coloration du plumage - Le pigeon biset est une des espèces montrant le plus fort taux de polymorphisme de coloration du plumage (Figure 5). Ce polymorphisme résulte de différences de taux de déposition d'eumélanine (pigmentation marron à noire) et de phéomélanine (pigmentation rousse). Parce que la synthèse de phéomélanine a un effet inhibiteur sur la synthèse d'eumélanine et vice-versa, les individus sont majoritairement eumélaniques ou phéomélaniques (Figure 7 ; Ducrest et al., 2008); ceci définit la nature de leur mélanisme. Ensuite, bien que la coloration du plumage du pigeon biset soit souvent catégorisée en morphes (« white », « blue-bar », « checker », « T-pattern », « spread » ; Figure 5), il existe une variation continue du degré de mélanisme du plumage allant du blanc au noir/roux. Malgré cette diversité

de colorations, certaines sont plus ou moins rares dans les populations. Ainsi, en Europe, les individus de couleurs intermédiaires (i.e. entre les morphes « checker » et « T-pattern ») sont les plus fréquents alors que les individus blancs (« white ») et phéomélaniques sont relativement rares (Obukhova, 2007).

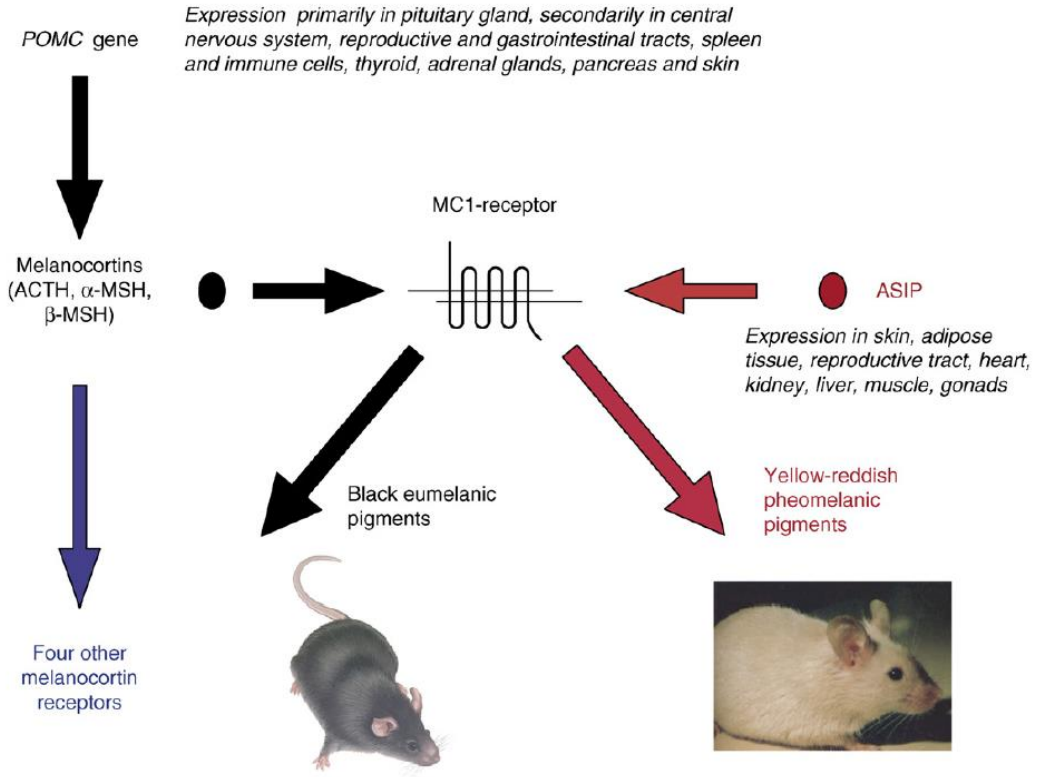


Figure 7. Synthèse de l'eumélanine et de la phéomélanine : la synthèse de phéomélanine a un effet inhibiteur sur la synthèse d'eumélanine et vice-versa (Ducrest et al. 2008).

Le pigeon biset a été choisi comme modèle biologique pour tester les effets écotoxicologiques des métaux traces et leur rôle dans le maintien du polymorphisme de couleur pour différentes raisons :

- son habitat urbain fait que le pigeon biset est fortement exposé aux métaux traces,
- la coloration mélanique de son plumage est très polymorphe,
- il s'acclimate facilement à la captivité et se reproduit bien en volières,
- il est très tolérant à la présence humaine et par conséquent le stress associé à la capture et au maintien en captivité est moindre par rapport à d'autres espèces d'oiseaux,

- il est de taille suffisante pour permettre des prélèvements sanguins permettant les mesures souhaitées,
- la biologie de cette espèce, ainsi que ses conditions de maintien en captivité et les signes renseignant sur son état de santé sont relativement bien connus.

LEXIQUE

Mélanocytes :	Cellules dérivées de la crête neurale et produisant les mélanosomes.
Mélanosomes :	Organite intracellulaire à l'intérieur desquels est synthétisée la mélanine.
Mélanine :	Pigment biologique responsable de la coloration brune, noire ou rousse des téguments (peau, poils, plumes) ; ces macromolécules sont produites par les mélanocytes.
Kératinocytes :	Cellules abondantes dans l'épiderme et les phanères, dont la différenciation et la migration (kératinisation) forme la kératine.
Kératine :	Protéine fibreuse responsable de la structure et de l'imperméabilité des téguments.

Capture et maintien en volière des pigeons

Les recherches que j'ai menées durant ma thèse ont porté sur des pigeons bisets capturés dans l'agglomération de Paris (France). Dès leur capture, les individus ont été identifiés individuellement à l'aide de bagues colorées et numérotées. Les individus ainsi capturés ont été transférés en volières extérieures au Centre d'Ecologie Expérimentale et Prédictive situé à Saint-Pierre-lès-Nemours (France), c'est-à-dire dans un environnement standardisé et rural. Les oiseaux ont été sexés, soit génétiquement, soit morphologiquement (i.e. grâce à une analyse discriminante) et répartis de façon à avoir 6 mâles et 6 femelles dans chaque volière.

Les oiseaux ont été nourris *ad-libitum* avec un mélange de maïs, de pois et de blé. Dans chaque volière étaient placées une soucoupe remplie d'eau que les oiseaux utilisent pour se baigner, ainsi que des perches. Par ailleurs, chaque volière disposait de 6 nichoirs (1/couple potentiel) dont l'accès pouvait être ouvert ou non aux pigeons (Image 1).

Une photographie de l'aile gauche et de l'aile droite a été faite sur chaque oiseau afin de mesurer la coloration de son plumage et ainsi estimer la concentration en mélanine dans les plumes (Image 2).

Image 1. Agencement d'une volière dans laquelle les pigeons étaient maintenus tout au long d'une étude.

Image 2. Photographie de l'aile gauche d'un pigeon. La surface bleutée correspond à la partie utilisée pour estimer le degré de mélanisme du plumage.

Résumé synthétique des études menées durant la thèse

Durant ma thèse, j'ai dans un premier temps mené une étude semi-corrélative (N.B. à partir d'échantillons préalablement récoltés) dans laquelle des pigeons bisets ont été capturés en périphérie de Paris et transférés en volières ; une fois en volière, les pigeons n'ont pas été supplémentés en métaux traces. J'ai par la suite mené deux études expérimentales dans lesquelles des pigeons bisets ont été capturés dans plusieurs populations parisiennes et transférés en volières ; ces pigeons ont alors été divisés en quatre traitements (Plomb, Zinc, Plomb+Zinc, Contrôle).

Etude	Date	N	Traitement	Mesures
Semi-corrélative	2009-2010	97	⌀	▪ Concentrations en zinc et plomb dans les plumes
Expérimentale 1	2013	96	- Plomb 1ppm - Zinc 10 ppm - Plomb 1ppm +Zinc 10 ppm - Contrôle	▪ Concentrations en zinc et en plomb dans les plumes, le sang, les œufs, le lait de jabot et les organes ▪ Immunité (anticorps spécifiques, réponse inflammatoire, parasites sanguins, réponse du complément) ▪ Corpulence ▪ Reproduction (qualité des œufs et des juvéniles) ▪ Communautés bactériennes du plumage (composition, abondance, richesse) ▪ Comportement (e.g. lissage) ▪ Profils leucocytaires ▪ Coloration mélanique des plumes ▪ Coloration iridescente des plumes ▪ Lysozymes et ovotransferrine des œufs ▪ Concentrations en mélanine dans le foie et les reins
Expérimentale 2	2014	144	- Plomb 10 ppm - Zinc 100 ppm - Plomb 10 ppm +Zinc 100 ppm - Contrôle	▪ Reproduction (qualité des œufs et des juvéniles) ▪ Hormone de stress (i.e. corticostérone) ▪ Hormones de soins parentaux (prolactine, testostérone, progéstérone) ▪ Lysozymes et ovotransferrine des œufs ▪ Concentration en zinc et en plomb dans les organes ▪ Concentrations en mélanine dans le foie et les reins

Validation du protocole d'exposition aux métaux traces (Etudes expérimentales 1 et 2)

Lors des études expérimentales 1 et 2, le zinc (sulfate de zinc) et le plomb (acétate de plomb), deux des métaux les plus abondants dans l'environnement urbain, ont été ajoutés dans l'eau de boisson et de bain des pigeons, et remplacée tous les deux jours jusqu' à la fin de l'expérience. Les concentrations en plomb ont été choisies sur la base des concentrations sanguines en plomb mesurées chez les oiseaux urbains (comprises entre 0,053 et 0,264 ppm ; Roux and Marra, 2007) et sur les taux d'absorption intestinale du plomb chez le diamant mandarin qui peuvent être calculés à partir de l'étude réalisée par Dauwe et ses collaborateurs (2002; <10%).

Lors de l'étude expérimentale 1, les concentrations en zinc et en plomb dans le sang et les plumes nouvellement formées (après leur retrait synchronisé 8 semaines après le début des traitements) ont été mesurées 10 et 12 semaines respectivement après le début des traitements, ceci dans le but de s'assurer de l'efficacité de notre protocole de supplémentation en métaux, c'est-à-dire que les métaux ajoutés dans l'eau ont été ingérés puis absorbés par les pigeons. Comme attendu, les concentrations en plomb dans le sang et dans les plumes étaient plus élevées chez les individus exposés au plomb par rapport aux non exposés (respectivement $F_{1,45}$=4,47, P=0.040 et $F_{1,76}$=19.61, P<0.001). De même, les concentrations sanguines en zinc étaient plus élevées chez les individus exposés au zinc que chez les non exposés ($F_{1,67}$=5.52, P=0.022). Aussi, comme voulu, les métaux ajoutés dans l'eau a permis d'augmenter significativement l'exposition des pigeons à ces métaux et leur absorption.

Bien que les concentrations en plomb et en zinc n'aient pas été mesurées dans le sang et les plumes des individus lors de l'étude expérimentale 2, les concentrations auxquelles ont été soumis les oiseaux étaient 10 fois supérieures à celle de l'étude expérimentale 1. Aussi, il apparait plus que raisonnable de penser que notre protocole de supplémentation ait été tout autant efficace, si ce n'est plus.

Chapitre 1 :

Rôle de la mélanine dans la fixation des métaux dans les plumes

Introduction

Des études *in vitro* ont mis en évidence le rôle chélateur de la mélanine. En effet, certains de ses groupements chargés négativement fixent les cations métalliques. Au moment de la croissance de la plume, les mélanosomes contenant les mélanocytes migrent du derme au germe de la plume via la circulation sanguine (voir Encadré 1) et avec eux migrent les ions métalliques fixés à la mélanine. Une fois sa croissance terminée, la plume se dévascularise et les éléments constituant la plume y restent donc piégés. Alors que beaucoup d'études se sont intéressées aux concentrations en métaux dans les plumes, notamment pour se servir des plumes comme bioindicateur de pollutions environnementales en métaux (Dauwe, L. Bervoets, R. Blust, M. Ee, 2002), peu d'études se sont intéressées aux raisons intrinsèques aux individus pouvant expliquer les variations de concentrations mesurées et plus particulièrement au rôle que pouvait jouer la mélanisation du plumage dans la séquestration des métaux. Ces études montrent d'ailleurs des résultats contradictoires et n'ont pas été menées dans des conditions standardisées, c'est-à-dire dans une situation dans laquelle tous les individus auraient été exposés aux mêmes concentrations environnementales en métaux (Dmowski et al., 1984; Gochfeld et al., 1991; Niecke et al., 1999). Or, les concentrations environnementales en métaux traces sont très variables, même à petite échelle spatiale (Frantz et al., 2012) et l'exposition des individus peut dépendre de facteurs qui leurs sont propres, comme leur sexe et leur âge (Agusa et al., 2005; Lucia et al., 2010), ou très probablement leur corpulence (déterminante de la quantité de nourriture qu'ils ingèrent) ou encore leur capacité cognitive ou leur statut social (déterminant de la qualité de la nourriture qu'ils ingèrent). Aussi, les études précédemment menées ne permettent pas de tester de façon précise le lien entre le degré de coloration mélanique du plumage et les concentrations en métaux traces.

Méthodes

Lors de l'étude semi-corrélative réalisée, les concentrations en zinc et en plomb ont été mesurées dans les plumes des pigeons, à la fois au moment de leur capture en milieu périurbain, et après un an de captivité dans un environnement standardisé rural.

Conclusion

Comme attendu, notre étude montre une corrélation positive entre le degré d'eumélanisme du plumage et la concentration en zinc dans les plumes. Ce résultat suggère que les individus au plumage le plus mélanique sont capables de séquestrer davantage de zinc dans leur plumage et que le transfert du zinc dans les plumes pourrait être un moyen de régulation des concentrations sanguines en zinc. Néanmoins, cette corrélation n'est mesurée qu'après un an de captivité ; à la capture, ni les concentrations en zinc, ni celles en plomb ne sont corrélées au degré d'eumélanisme du plumage. Ce résultat confirme notre hypothèse selon laquelle, en milieu naturel, les individus sont exposés à des concentrations environnementales en métaux différentes. Il souligne l'importance de recourir à la standardisation de l'exposition aux métaux traces afin de tester le rôle de la mélanine du plumage dans la fixation des métaux traces dans les plumes, mais aussi dans la détoxication.

Certains auteurs suggèrent que la corrélation positive mesurée entre le degré de mélanisme du plumage et les concentrations en certains métaux dans les plumes (e.g. zinc et calcium) résulterait du rôle essentiel de ces métaux dans la synthèse de mélanine (Niecke et al., 1999) et non pas de la capacité de la mélanine à fixer ces ions métalliques. Néanmoins, nous n'avons pas mesuré de corrélation entre le degré d'eumélanisme du plumage et les concentrations en zinc à la capture. De plus, le rôle potentiel de ces métaux dans la mélanogénèse influencerait davantage l'intensité de la coloration eumélanique (McGraw, 2006, 2003) que le pattern de coloration, qui lui, est déterminé de façon génétique (Jacquin et al., 2013; Roulin, 2004). Par exemple, la supplémentation en calcium augmente l'intensité de la coloration noire du plumage chez le diamant mandarin (McGraw, 2006). Lors de l'étude expérimentale 1 dans laquelle des pigeons bisets ont été exposés à du plomb et/ou du zinc, la supplémentation en zinc n'avait pas d'effet significatif sur l'intensité de la coloration noire du plumage (N.B. résultats n'ayant pas fait l'objet d'un manuscrit). Bien que l'hypothèse avançant le rôle de certains métaux dans la synthèse de la mélanine ne soit pas à écarter, elle explique difficilement la corrélation entre le degré d'eumélanisme du plumage et la concentration en zinc mesurée dans notre étude.

Du fait de concentrations en plomb après un an de captivité trop faibles dans les plumes, il n'a pas été possible de mettre en évidence de lien entre ces concentrations et le degré d'eumélanisme du plumage. Dans le but d'obtenir des concentrations en plomb dans les plumes détectables, il apparait nécessaire d'exposer les individus à des concentrations suffisantes et standardisées de plomb. Par ailleurs, des études similaires doivent être conduites afin de pouvoir généraliser le rôle chélateur de la mélanine du plumage à l'ensemble des métaux traces et, de

façon encore plus intéressante dans le cadre de notre étude, aux métaux traces fortement toxiques comme le plomb et le cadmium.

Les individus au plumage le plus mélanique séquestrant davantage de zinc et potentiellement d'autres métaux dans leurs plumes, il est fort à parier que le degré d'eumélanisme du plumage détermine en partie les concentrations internes (i.e. dans le sang et les organes) en métaux traces. Tester le lien entre le degré d'eumélanisme du plumage et les concentrations en métaux dans les plumes, le sang et les organes apparait être l'étape suivante essentielle à la mise en évidence d'un rôle détoxifiant de la mélanine du plumage.

Ce chapitre prend la forme d'un article publié dans Biology Letters

biology letters

rsbl.royalsocietypublishing.org

Research

Cite this article: Chatelain M, Gasparini J, Jacquin L, Frantz A. 2014 The adaptive function of melanin-based plumage coloration to trace metals. *Biol. Lett.* **10**: 20140164.
http://dx.doi.org/10.1098/rsbl.2014.0164

Received: 20 February 2014
Accepted: 10 March 2014

Subject Areas:
ecology, evolution, environmental science, health and disease and epidemiology

Keywords:
melanin-based coloration, trace metals, urban wildlife, polluted environment

Author for correspondence:
M. Chatelain
e-mail: marion.chatelain@snv.jussieu.fr

Electronic supplementary material is available at http://dx.doi.org/10.1098/rsbl.2014.0164 or via http://rsbl.royalsocietypublishing.org.

Royal Society Publishing

Evolutionary biology

The adaptive function of melanin-based plumage coloration to trace metals

M. Chatelain[1], J. Gasparini[1,2], L. Jacquin[1,3,4] and A. Frantz[1,2]

[1]UPMC Univ Paris 06, and [2]CNRS, Institut d'Ecologie et des Sciences de l'Environnement, Paris, France
[3]UMR 1224, ECOBIOP, Université de Pau et des Pays de l'Adour UPPA, Pôle d'Hydrobiologie, Saint-Pée sur Nivelle, France
[4]UMR 1224, ECOBIOP, INRA Aquitaine, Pôle d'Hydrobiologie, Saint-Pée-sur-Nivelle, France

Trace metals produced by anthropogenic activities are of major importance in urban areas and might constitute a new evolutionary force selecting for the ability to cope with their deleterious effects. Interestingly, melanin pigments are known to bind metal ions, thereby potentially sequestering them in inert body parts such as coat and feathers, and facilitating body detoxification. Thus, a more melanic plumage or coat coloration could bring a selective advantage for animals living in polluted areas. We tested this hypothesis by investigating the link between melanin-based coloration and zinc and lead concentrations in feathers of urban feral pigeons, both at capture time and after one year of captivity in standardized conditions. Results show that differently coloured pigeons had similar metal concentrations at capture time. Metal concentrations strongly decreased after one year in standardized conditions, and more melanic pigeons had higher concentrations of zinc (but not lead) in their feathers. This suggests that more melanic pigeons have a higher ability to store some metals in their feathers compared with their paler counterparts, which could explain their higher success in urbanized areas. Overall, this work suggests that trace metal pollution may exert new selective forces favouring more melanic phenotypes in polluted environments.

1. Introduction

Trace metals are bioavailable chemicals found in very small concentrations in the environment. They are emitted by natural processes [1,2] and by anthropogenic activities [3]. Consequently, their concentrations are much higher in urban environments and urban wildlife populations than in their rural counterparts [3–5]. Recent correlative and experimental studies have shown that some trace metals affect individual fitness by impairing reproductive success and survival [6–13]. For example, bird populations exposed to higher trace metal levels have a reduced clutch size [12] and impaired male fertility [9]. Because of this, trace metals are likely to exert new selective pressures on wildlife in urban areas.

Such evolutionary pressures may select for individuals able to detoxify their body or tolerate large amounts of trace metals. Because the property of melanin to bind metal ions may allow some body detoxification, we hypothesized that more melanic plumage may be selected in urban environments. Indeed, melanin is composed of several polymers with negatively charged free carboxyl, hydroxyl and amine functions which bind metal ions *in vitro* [14–16] explaining the relation found between some metals and melanic pigmentation [17]. Therefore, highly melanic birds could transfer more metals to their growing feathers during moult, thereby detoxifying their blood at a higher rate in comparison with less melanic individuals. First, this hypothesis predicts that an environment with a high level of trace metals, such as urban habitat, would select for highly melanic individuals. In agreement with this, the degree of melanin-based coloration correlates with the degree of urbanization in feral pigeons from European cities [18,19].

Secondly, this hypothesis predicts that, in a standardized environment, the concentration of trace metals in the feathers should be positively related to the degree of individual melanin-based coloration. To test this prediction, we investigated the link between zinc and lead concentrations and the degree of melanin-based coloration in feral pigeon's feathers in metropolitan Paris, France, where zinc and lead are two of the most widespread environmental metals contaminants [3].

2. Material and methods

A total of 97 (53 male and 44 female) eumelanic adult free-living feral pigeons (*Columba livia*) were caught in February 2009 at two locations of the Parisian suburbs (Courbevoie and Gennevilliers) and randomly distributed with respect to origin, gender and coloration in 10 outdoor aviaries (2.20 m × 2.20 m) at the CEREEP field station (Centre d'Ecologie Expérimentale et Prédictive-Ecotron Ile-de-France, UMS 3194, Ecole Normale Supérieure, Saint-Pierre-lès-Nemours, France) in standardized conditions. Birds were fed ad libitum with a mix of maize, wheat and peas. The aviaries were enriched with a bowl of water used for bathing and with branches as perches. At capture time, birds were individually identified with a numbered colour plastic ring and photographed to measure the degree of coloration: we calculated a continuous coloration score as the percentage of dark surface on the wing which is a reliable and repeatable index [20]. Two innermost greater secondary coverts were collected on each wing and stored in metal-free polyethylene bags until metal concentration measurements. The two same new-grown feathers were collected one year later (February 2010).

Zinc (Zn) and lead feather concentrations were measured for all individuals at capture and after one year of captivity. Feathers were mineralized as described in Frantz *et al*. [21]. Feathers were washed vigorously with 0.25 M NaOH solution, rinsed energetically three times in ultrapure water (Milli-Q purified) to remove external contamination [5], left for 1 h in ultrapure water, dried for 12 h at 80°C to dry mass, crushed to powder and weighed to the nearest 0.1 mg. Feathers were then digested twice in 1 ml nitric acid (67%), followed by a final digestion in 1 ml hydrogen peroxide (30%).

Total Zn concentrations were determined using flame atomic absorption spectrometry (FAAS, Unicam AA Series Spectrometer, Thermo Electron Corporation) and total Pb concentrations using graphite furnace spectrometry (ETAAS, Unicam 989 QZ AA Spectrometer, Zeeman SOLAAR). For each sample, three values measured for each metal were averaged when the relative standard deviation was less than 5%.

Statistical analyses were performed using R (R v. 2.12.0). To test for a relationship between the degree of coloration and the concentrations of trace metals in the feathers, we used two independent mixed linear models with zinc or lead as the dependent variable, and coloration score, collection time (capture time versus one year later) and their interaction as fixed factors. Capture site and individual identity were also included as random effects in the models.

3. Results

Zinc and lead concentrations were highly correlated at capture time (Pearson's correlation test: $r = 0.62$, $t_{\text{d.f.}= 90} = 7.52$, $p < 0.001$; figure 1). This positive relation disappeared one year later (Pearson's correlation test: $r = 0.12$, $t_{\text{d.f.}= 87} = 1.05$, $p = 0.297$).

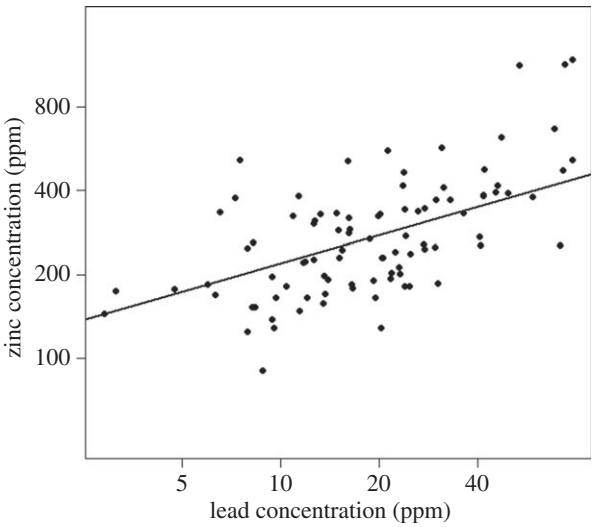


Figure 1. Correlation between zinc and lead concentrations (ppm) in the feathers at capture time.

Lead concentration in the feathers decreased after one year of captivity (table 1; respectively, 24.340 ppm ± 18.078 and 0.706 ppm ± 0.421), but was not related to coloration score (table 1).

Zinc concentration in the feathers also decreased after one year of captivity (table 1; respectively, 328 ppm ± 238 and 89 ppm ± 30). Moreover, zinc concentration was significantly affected by the interaction between coloration score and collection time (table 1 and figure 2): while differently coloured pigeons had similar zinc concentration in their feathers at capture time (ANOVA: $\chi^2 = 0.36$, d.f. = 86, $p = 0.551$), more melanic pigeons had higher zinc concentration in their feathers than paler ones after one year of captivity (ANOVA: $\chi^2 = 24.94$, d.f. = 84, $p < 0.001$).

4. Discussion

Results showed that darker individuals had a higher zinc burden in their feathers compared with paler ones when kept in standardized conditions. This result is consistent with the hypothesis that more pigmented individuals can store higher amounts of metal ions.

This relation was only found after one year of captivity. Differently coloured birds were exposed to the same environment, and metal availability was consequently similar, suggesting that the difference between morphs was not due to different uptake rates. Some environmental factors may have hidden the correlation between coloration and metal concentrations at capture time. Although birds were captured from closely located sites, we cannot exclude local differences in pollution levels, known to change even at a small scale [1,21]. Moreover, habitat use may vary among differently coloured pigeons, as is often the case in polymorphic species [22]. For instance, darker pigeons might exploit less polluted areas but have a greater ability to store metals in their feathers than paler pigeons, which could result in comparable levels of metals in dark and pale pigeons at capture time.

In contrast to zinc, we did not find any correlation between feather lead concentration and melanin-based coloration score. It is likely that the very small amount and low variation of

Table 1. Final mixed linear model ANOVAs with log-transformed zinc or lead concentration as the dependant variable in separate models, coloration score and time of collection as covariates, and capture site and ring name as random effects. Lead concentration was neither explained by the interaction between coloration score and time of collection ($\chi^2 = 0.33$, d.f. = 81, $p = 0.568$) nor by coloration score as simple effect ($\chi^2 = 0.42$, d.f. = 90, $p = 0.519$) and were removed from the model. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

	zinc concentration			lead concentration		
	χ^2	d.f.	p	χ^2	d.f.	p
coloration score	0.536	88	0.464			
time of collection	247.069	85	<0.001***	1288.2	82	<0.001***
coloration score × time	8.188	85	0.004**			

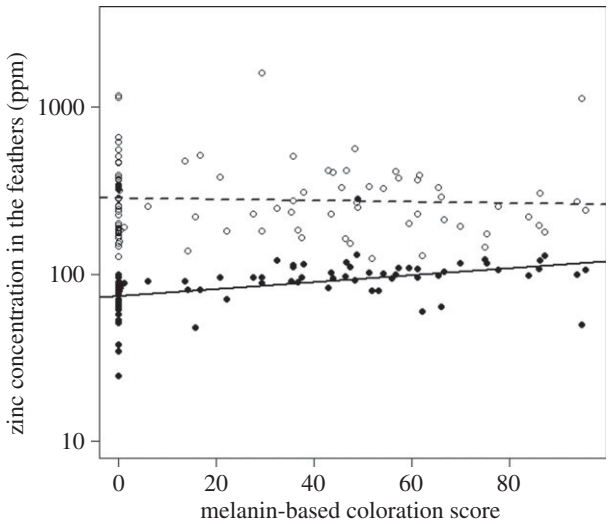


Figure 2. Relationship between the degree of melanin-based coloration and concentration of zinc in the feathers (ppm) at the time of capture (open circles) and after 1 year of captivity (filled circles).

lead concentrations in feathers after one year of captivity do not allow detection of any relationship. However, the elevated values of lead concentrations in feathers collected at capture time confirm that feathers do sequester lead [5,21]. Thus, we cannot exclude the possibility that feathers constitute a way to detoxify vital organs of lead as well as zinc.

In conclusion, this correlative study sheds light on the link between melanin-based coloration and metal concentrations in feathers. Obviously, melanin could have evolved for several reasons such as parasite resistance [20], camouflage [23] or thermoregulation [24]. Nevertheless, this study suggests that trace metals could represent a current selective force favouring melanic phenotypes among urban organisms. Such selection could explain the higher frequency of more melanic birds in urban habitats [18,19]. Experimental manipulations of trace metal exposure are now required, to first test whether melanism enables maintenance of reduced metal concentrations in blood, then to compare fitness traits between differently coloured individuals. In particular, the effectiveness of detoxification via melanic feathers may depend on moulting intensity and level of metal intake.

All experiments were carried out in strict accordance with the recommendations of the 'European Convention for the Protection of vertebrate Animals used for Experimental and Other Scientific Purposes' and were conducted under the authorization of the 'Direction Départementale des Services Vétérinaires de Seine et-Marne' (permit no. 77-05).

Acknowledgements. We are very grateful to Bioemco laboratory and more particularly to E. Aubry and M. Castrec-Rouelle for their assistance to trace metal measurements. We thank Julie Legoupi for her involvement in the methodological part. We thank the 'Mairie de Paris' (Thomas Charachon) for allowing the capture of birds and the CEREEP field station where birds were maintained.

Data accessibility. Raw data are provided in the electronic supplementary material.

References

1. Nriagu JO. 1989 A global assessment of natural sources of atmospheric trace metals. *Nature* **338**, 47–49. (doi:10.1038/338047a0)
2. Pongratz R, Heumann KH. 1999 Production of methylated mercury, lead, and cadmium by marine bacteria as a significant natural source for atmospheric heavy metals in polar regions. *Chemosphere* **39**, 89–102. (doi:10.1016/S0045-6535(98)00591-8)
3. Azimi S, Rocher V, Muller M, Moilleron R, Thevenot D. 2005 Sources, distribution and variability of hydrocarbons and metals in atmospheric deposition in an urban area (Paris, France). *Sci. Total Environ.* **337**, 223–239. (doi:10.1016/j.scitotenv.2004.06.020)
4. Roux K, Marra P. 2007 The presence and impact of environmental lead in passerine birds along an urban to rural land use gradient. *Arch. Environ. Contam. Toxicol.* **53**, 261–268. (doi:10.1007/s00244-006-0174-4)
5. Scheifler R *et al.* 2006 Lead concentrations in feathers and blood of common blackbirds (*Turdus merula*) and in earthworms inhabiting unpolluted and moderately polluted urban areas. *Sci. Total Environ.* **371**, 197–205. (doi:10.1016/j.scitotenv.2006.09.011)
6. Rainbow P. 2007 Trace metal bioaccumulation: models, metabolic availability and toxicity. *Environ. Int.* **33**, 576–582. (doi:10.1016/j.envint.2006.05.007)
7. Hsu MJ, Selvaraj K, Agoramoorthy G. 2006 Taiwan's industrial heavy metal pollution threatens terrestrial biota. *Environ. Pollut.* **143**, 327–334. (doi:10.1016/j.envpol.2005.11.023)
8. Trust K, Miller M, Ringelman J, Orme I. 1990 Effects of ingested lead on antibody production in mallards (*Anas platyrhynchos*). *J. Wildl. Dis.* **25**, 316–322. (doi:10.7589/0090-3558-26.3.316)

9. Dauwe T, Janssens E, Kempenaers B, Eens M. 2004 The effect of heavy metal exposure on egg size, eggshell thickness and the number of spermatozoa in blue tit *Parus caeruleus* eggs. *Environ. Pollut.* **129**, 125–129. (doi:10.1016/j.envpol.2003.09.028)
10. Greenberg S, Briemberg H. 2004 A neurological and hematological syndrome associated with zinc excess and copper deficiency. *J. Neurol.* **251**, 111–114. (doi:10.1001/s00415-004-0263-0)
11. Snoeijs T, Dauwe T, Pinxten R, Vandesande F, Eens M. 2004 Heavy metal exposure affects the humoral immune response in a free-living small songbird, the great tit (*Parus major*). *Arch. Environ. Contam. Toxicol.* **46**, 399–404. (doi:10.001/s00244-003-2195-6)
12. Eeva T, Ahola M, Lehikoinen E. 2009 Breeding performance of blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*) in a heavy metal polluted area. *Environ. Pollut.* **157**, 3126–3131. (doi:10.1016/j.envpol.2009.05.040)
13. Plum L, Rink L, Haase H. 2010 The essential toxin: impact of zinc on human health. *Int. J. Environ. Res. Public Health* **7**, 1342–1365. (doi:10.3390/ijerph7041342)
14. Larsson B, Tjälve H. 1978 Studies on the mechanism of drug-binding to melanin. *Biochem. Pharmacol.* **28**, 1181–1187. (doi:10.1016/0006-2952(79) 90326-5)
15. Liu Y, Hong L, Kempf V, Wakamatsu K, Ito S, Simon J. 2004 Ion-exchange and adsoption of Fe(III) by *Sepia* melanin. *Pigment Cell Res.* **17**, 262–269. (doi:10.1111/j.1600-0749.2004.00140.x)
16. Bridelli M, Crippa P. 2008 Theoretical analysis of the adsorption of metal ions to the surface of melanin particles. *Adsorption* **14**, 101–109. (doi:10.007/s10450-007-9059-8)
17. Niecke M, Rothlaender S, Roulin A. 2003 Why do melanin ornaments signal individual quality? Insights from metal element analysis of barn owl feathers. *Oecologia* **137**, 153–158. (doi:10.1007/s00442-003-1307-3)
18. Obukhova N. 2007 Polymorphism and phene geography of the blue rock pigeon in Europe. *Russ. J. Genet.* **43**, 492–501. (doi:10.1134/S1022795407050031)
19. Jacquin L, Récapet C, Prévot-Julliard A, Leboucher G, Lenouvel P, Erin N, Corbel H, Frantz A, Gasparini J. 2013 A potential role for parasites in the maintenance of color polymorphism in urban birds. *Oecologia.* (doi:10.007/s00442-013-2663-2)
20. Jacquin L, Lenouvel P, Haussy C, Ducatez S, Gasparini J. 2011 Melanin-based coloration is related to parasite intensity and cellular immune response in an urban free living bird: the feral pigeon *Columba livia*. *J. Avian Biol.* **42**, 1–5. (doi:10.1111/j.1600-048X.2010.05120.x)
21. Frantz A, Pottier MA, Karimi B, Corbel H, Aubry E, Haussy C, Gasparini J, Castrec-Rouelle M. 2012 Contrasting levels of heavy metals in the feathers of urban pigeons from close habitats suggest limited movements at a restricted scale. *Environ. Pollut.* **168**, 23–28. (doi:10.1016/j.envpol.2012.04.003)
22. Roulin A. 2004 The evolution, maintenance and adaptive function of genetic colour polymorphism in birds. *Biol. Rev.* **79**, 815–848. (doi:10.1017/S1464793104006487)
23. Broom M, Ruxton GD. 2005 You can run-or you can hide: optimal strategies for cryptic prey against pursuit predators. *Behav. Ecol.* **16**, 534–540. (doi:10.1093/beheco/ari024)
24. Walsberg G, Campbell G, King J. 1978 Animal coat color and radiative heat gain: a re-evaluation. *J. Comp. Physiol. B* **126**, 211–222. (doi:10.1007/BF00688930)

Chapitre 2 :

Rôle adaptatif de la mélanine face aux métaux traces

Introduction

Comme précédemment présenté, notre hypothèse propose que la mélanine du plumage est capable de fixer les ions métalliques. En accord avec nos prédictions, notre étude précédente (voir Chapitre 1) a mis en évidence une corrélation positive entre le degré d'eumélanisme du plumage et la concentration en zinc dans les plumes. Néanmoins, nous n'avons pas pu montrer une telle relation concernant le plomb, un métal toxique dont la détoxication via son transfert dans les plumes pourrait être nettement bénéfique ; la mise en évidence du rôle de la mélanine du plumage dans la séquestration de métaux hautement toxiques, par opposition au zinc qui l'est peu, apparaît donc nécessaire à la validation de notre hypothèse. Les résultats de notre première étude ont soulignés l'importance de tester le lien entre la coloration mélanique du plumage et sa capacité à séquestrer les métaux traces dans des conditions standardisées, c'est-à-dire en s'affranchissant des différences individuelles d'exposition (voir Chapitre 1).

De telles conditions sont également essentielles pour montrer le rôle détoxifiant de la mélanine du plumage via le transfert des métaux traces de la circulation sanguine dans les plumes lors de la mue, et, en conséquence, le lien entre le mélanisme du plumage et la sensibilité des individus aux métaux traces.

La corpulence, estimant l'état de santé de l'individu, et le succès reproducteur saisonnier sont deux paramètres biologiques susceptibles de refléter les effets physiologiques engendrés par les expositions aux métaux traces. En effet, plusieurs études corrélatives ont mis en évidence une altération de certains paramètres de la reproduction dans des habitats pollués en métaux traces (Dauwe et al., 2004; Eeva et al., 2009; Eeva and Lehikoinen, 1996; Sens et al., 2003). De plus ces deux paramètres biologiques semblent être les plus adaptés pour estimer les effets des métaux traces sur l'aptitude phénotypique des individus. Néanmoins, à notre connaissance, aucune étude expérimentale n'a été menée pour vérifier ces effets, pourtant susceptible d'affecter le fonctionnement et la dynamique des populations.

Méthodes

Lors de l'étude expérimentale réalisée en 2013 (étude expérimentale 1), j'ai mesuré les concentrations en plomb et en zinc dans le sang et les plumes des individus après respectivement 10 semaines et 13 semaines de traitements (N.B. une première plume a été retirée 9 semaines après le début des traitements et les métaux ont été dosés dans la plume nouvellement formée), ceci afin de mesurer la capacité de transfert des métaux dans les plumes et de détoxication, en fonction du degré de mélanisme du plumage. Par ailleurs, deux paramètres biologiques ont été mesurés dans le but d'estimer l'aptitude phénotypique des individus ; d'une part, j'ai mesuré de façon hebdomadaire le poids des individus afin d'étudier la variation d'un indice de corpulence au cours de l'expérience. D'autre part, j'ai suivi la reproduction des individus et mesuré différents paramètres de reproduction que sont le succès d'accouplement, la qualité des œufs (i.e. le poids et le volume chez l'ensemble des œufs, ainsi que le poids du jaune, du blanc et de la coquille et l'épaisseur de la coquille chez les œufs de la 2nd et 4ème ponte qui ont été retirés et congelés), le succès d'éclosion, la croissance des juvéniles de leur éclosion jusqu'à environ 25 jours, le succès d'envol et la qualité des juvéniles à 3 mois (i.e. leur indice corporel, leur taux d'hématocrite et leur profil leucocytaire).

Conclusions

Pour la première fois, notre étude met en évidence une corrélation positive entre le degré d'eumélanisme du plumage et la concentration en plomb et en zinc dans les plumes. Ce résultat suggère que le degré d'eumélanisme du plumage reflète la capacité des individus à séquestrer les métaux traces. En effet, alors que le zinc pourrait constituer un élément essentiel à la synthèse de la mélanine, expliquant de ce fait le lien entre ses concentrations et le degré de mélanisme du plumage, le plomb est un xénobiotique vrai.

Par ailleurs, les individus eumélaniques montraient des concentrations en zinc dans les plumes plus élevées que les individus phéomélaniques. Cette différence souligne l'importance des groupes carboxyles dans la fixation de certains métaux traces, tout du moins du zinc, plus nombreux dans la molécule d'eumélanine que de phéomélanine. Puisque les concentrations sanguines en zinc ne diffèrent pas entre les individus eumélaniques et phéomélaniques, nous pouvons penser que les mécanismes de régulation des concentrations internes en zinc ou que les besoins physiologiques en cet élément diffèrent selon la coloration mélanique du plumage.

Malgré le rôle de la mélanine du plumage dans la séquestration du plomb et du zinc, les individus les plus mélaniques ne maintenaient pas des concentrations sanguines plus faibles par

rapport aux individus plus clairs. Alors que ce résultat pouvait être attendu dans le cas du zinc si l'on considère que ses concentrations sanguines sont régulées de façon homéostatique, ce n'était pas le cas pour le plomb. D'une part, cela peut signifier que le transfert des métaux dans les plumes est trop faible par rapport à son apport continu via l'eau de boisson et de bain. Chez le pigeon biset, la mue est quasiment continue au cours de l'année mais présente un pic à l'automne. Par ailleurs, chez les juvéniles, les plumes poussent de façon simultanée. Dans ces deux conditions, il est possible que le transfert des métaux dans les plumes soit suffisant à la diminution des concentrations internes en métaux traces. D'ailleurs, la détoxication serait particulièrement bénéfique à ces deux périodes de la vie des individus, en diminuant l'investissement dans des mécanismes de détoxication actifs coûteux (e.g. enzymes de détoxication) dans des périodes où le taux de mortalité est le plus élevé (Johnston and Janiga, 1995).

Néanmoins, il est également probable que les concentrations sanguines ne soient pas de bons indicateurs de l'exposition récente aux métaux traces. En effet, les métaux absorbés sont accumulés dans les organes internes, notamment les organes filtreurs que sont les reins et le foie, et le squelette, et peuvent être relargués secondairement dans la circulation sanguine (Agusa et al., 2005; Cosson et al., 1988; Gulson et al., 1996; Kim et al., 1998) ; ce relargage dépend des quantités accumulées, de la porosité des os et éventuellement d'autres facteurs intrinsèques aux individus (Gulson et al., 1996). Aussi, les concentrations sanguines en métaux reflètent une combinaison des expositions présentes et passées. Il apparait relativement difficile de mesurer la proportion des métaux ingérés durant l'expérience accumulée dans les parties vivantes des individus. Il faudrait pour cela mener une expérience similaire à la nôtre mais dans laquelle les individus utilisés n'auraient jamais été exposés à des métaux traces toxiques (i.e. montrant des concentrations initiales en métaux proches de zéro) et mesurer les concentrations en métaux dans leur sang mais aussi dans leurs organes.

Les concentrations en zinc et en plomb mesurées à la fois dans le sang et les plumes des individus, en plus de vérifier notre protocole expérimental, c'est-à-dire l'absorption des métaux traces (voir « Méthodes »), révèlent certaines interférences entre les métabolismes du zinc et du plomb. En effet, nous avons mesuré des concentrations sanguines en plomb plus faibles chez les individus exposés au zinc seul, probablement la conséquence du rôle du zinc dans la diminution de l'absorption gastro-intestinale du plomb, connu chez les mammifères (Cerklewski and Forbes, 1976; El-Gazzar et al., 1978).

Notons également que les concentrations sanguines en zinc étaient plus faibles dans notre expérience que celles mesurées à la capture (respectivement 3,96ppm±0,17 5,02ppm±0,06). Or, durant notre expérience, du zinc était transféré dans les plumes ; ceci suggère que les concentrations sanguines en zinc ne sont pas strictement régulées et que par ailleurs, soit, en

milieu urbain, les concentrations en zinc dans l'environnement sont tellement élevées que les mécanismes de régulations sont insuffisants, soit, en volières, les individus sont carencés et alors le transfert de zinc dans les plumes pourrait avoir des effets nocifs, soit encore les besoins basaux en zinc sont différents en milieu urbain et rural et il existe une régulation des concentrations sanguines en zinc qui est fonction des besoins. Comprendre les mécanismes de métabolisation des métaux traces essentiels apparaît indispensable à la compréhension du rôle bénéfique ou non du transfert de ces éléments dans le plumage.

Peut-être du fait que l'efficacité de la détoxication des métaux ne dépende pas du mélanisme des individus, la tolérance aux métaux traces semble peu dépendre de la coloration mélanique du plumage ; seul le maintien de la corpulence au cours de l'expérience dépend de l'interaction entre l'exposition aux métaux et le degré de mélanisme du plumage. En effet, chez les pigeons exposés au plomb seul et contrairement aux individus les plus clairs, les individus davantage eumélaniques ont perdu du poids au cours de l'expérience. Néanmoins, il nous est difficile d'interpréter ce résultat. D'une part, cet effet peut résulter d'un biais initial (i.e. il se trouve qu'en début d'expérience les individus davantage mélaniques étaient moins corpulents). Par ailleurs, dans le cas où cet effet ne découlerait pas d'un biais, il pourrait d'une part suggérer un désavantage à l'eumélanisme dans un environnement pollué en métaux traces, ce qui serait alors contraire à notre hypothèse, ou souligner un compromis entre le maintien de la corpulence et d'autres fonctions biologiques. Entre autres, les individus dont le plumage présente un fort degré d'eumélanisme pourraient s'être davantage investis dans la reproduction comme le suggère la plus forte fréquence de juvéniles au plumage foncé chez les individus exposés au plomb seul. Ce dernier point, et malgré l'absence de résultats clairs sur le rôle bénéfique de l'eumélanisme chez les adultes exposés au plomb, suggère une survie préférentielle des juvéniles les plus foncés. Comme suggéré plus haut, les juvéniles, dont la croissance du plumage est synchronisée, pourraient montrer une plus grande capacité à se détoxifier à un moment déterminant pour leur survie et leur croissance. Néanmoins, l'avantage des juvéniles les plus foncés peut également résulter des effets génétiques liant le mélanisme du plumage à l'immunité, la résistance au stress oxydatif, etc. (Ducrest et al., 2008; Jacquin et al., 2011; Roulin et al., 2011). Afin de dissocier ces deux hypothèses, et bien que cela soit difficile, il serait intéressant d'estimer le taux de détoxication des juvéniles en fonction de la coloration mélanique de leur plumage. Quel que soit le mécanisme impliqué, ce résultat est en accord avec une étude corrélative précédemment menée dans une population suburbaine de pigeons bisets et montrant une meilleure survie des juvéniles les plus foncés (Récapet et al., 2013), ainsi qu'avec la plus forte fréquence des pigeons foncés en ville par rapport au milieu rural (Obukhova, 2007).

Notre étude confirme certains effets écotoxicologiques associés à une exposition aux métaux traces observés *in natura*. En effet, l'exposition au plomb semble altérer plusieurs paramètres de la reproduction, entre autres en diminuant la qualité de la croissance des juvéniles (i.e. poids à l'éclosion, durée de la croissance, corpulence à 3 mois) et leur survie (i.e. succès d'envol), et en augmentant leur stress physiologique (i.e. ratio hétérophiles/lymphocytes). Au contraire, l'exposition au zinc apparait bénéfique au maintien de la corpulence au cours de l'expérience, à la qualité des œufs (i.e. poids du jaune et épaisseur de la coquille) et des juvéniles (i.e. nombre de globules blancs). De plus, le zinc semble avoir un effet protecteur chez les individus également exposés au plomb, ceux-ci ne montrant pas les symptômes observés chez les individus exposés au plomb seul (i.e. altération de la durée de la croissance et de la corpulence à 3 mois, du succès d'envol et du stress physiologique). Cet effet protecteur est susceptible de résulter du rôle du zinc dans la diminution de l'absorption gastro-intestinale du plomb évoquée précédemment (Cerklewski and Forbes, 1976; El-Gazzar et al., 1978). En effet, l'effet du zinc sur les concentrations sanguines en plomb semble se répercuter sur le transfert du plomb aux juvéniles, via les œufs et le lait de jabot ; les œufs pondus par des femelles exposées au zinc et le lait de jabot prélevé chez les poussins dont les parents étaient exposés au zinc présentent des concentrations en plomb plus faibles par rapport aux œufs ($F_{1,16}$=9.85, P=0,040; respectivement 12,8±6,3 et 153,0±122,4 ppb) et au lait de jabot (F1,18=3,35, P=0,063; respectivement 0,7±0,2 et 2,3±1,0 ppm) des parents non exposés au zinc (N.B résultats n'ayant pas fait l'objet d'un manuscrit). Ces résultats soulèvent l'importance de considérer l'exposition aux métaux traces comme une exposition à un assemblage de métaux susceptibles d'avoir des effets antagonistes ou synergiques. L'effet global d'une telle exposition dépend alors de la proportion de chacun des métaux.

Les concentrations en zinc et en plomb dans les plumes étant respectivement 80 et 1,5 fois plus faibles dans notre expérience que celles mesurées chez des individus capturés en milieu urbain (Adout et al., 2007; Frantz et al., 2012; Brait and Filho, 2011; Nam et al., 2004), les effets mesurés dans notre expérience risquent d'être beaucoup plus marqués en milieu urbain. Bien que les paramètres mesurés dans notre étude ne soient qu'une approximation de l'aptitude phénotypique des individus, notre étude soulève les risques que les métaux traces sont susceptibles de représenter sur les populations urbaines. Il est clair que des études futures devront mesurer la survie et le succès reproducteur à long terme des individus afin d'estimer les effets des métaux sur le fonctionnement et la dynamique des populations.

Ce chapitre prend la forme d'un article soumis dans The American Naturalist.

Does trace metals select for darker birds in urban areas?

An experimental exposure to lead and zinc

Chatelain M[1], Gasparini J[1] and Frantz A[1]

[1] Sorbonne Universités, UPMC Univ Paris 06, UPEC, Paris 7, CNRS, INRA, IRD, Institut d'Ecologie et des Sciences de l'Environnement de Paris, F-75005, Paris, France

Address for correspondence: Université Pierre et Marie Curie, Institut d'Ecologie et des Sciences de l'Environnement, 7 quai St Bernard 75005 Paris, France

Running title: trace metals and plumage melanin selection

Table: 1

Figures: 5

ABSTRACT

Trace metals from anthropogenic activities are involved in numerous health impairments and therefore may select for detoxification mechanisms or a higher tolerance. Melanin, responsible for the black and red colourations of teguments, plays a role in metal ions chelation and its synthesis is positively linked to immunity, to antioxidant capacity and to stress resistance due to pleiotropic effects. Therefore, we expected darker birds to 1) store higher amounts of metals into their feathers, 2) maintain lower metal blood concentrations and 3) suffer less from metal exposure. We exposed feral pigeons (*Columba livia*) exhibiting various plumage darkness levels to low but chronic concentrations of zinc and/or lead, two of the most abundant metals in urban areas. First, we found negative and positive effects of lead and zinc respectively on birds' condition and reproductive parameters. Then, we observed positive relationships between plumage darkness and both zinc and lead concentrations. Interestingly, while darker adults did not maintain lower metal blood concentrations and did not have higher fitness parameters, darker juveniles exhibited a higher survival rate than paler ones when exposed to lead. Our results suggest that trace metals may favour darker birds and may therefore be involved in melanin-based plumage polymorphism maintenance.

Key words: ecotoxicology, urban pollution, plumage colouration, eumelanin, pheomelanin

INTRODUCTION

Anthropogenic activities are responsible for large environmental modifications including luminous, noise and chemical pollutions that may have consequences on populations' dynamics and functioning. These environmental factors may exert new selective pressures, especially in urban areas in which human activities likely have the most drastic impacts. Trace metals are of particular great concern, given their implication in several human diseases (reviewed in Jarup 2003) and their noxious effect on biodiversity (Hsu et al. 2006). For instance, trace metals negatively impact immunity in great tits and zebra finches (Snoeijs et al. 2004; Snoeijs et al. 2005), and learning abilities in young herring gulls (Burger and Gochfeld 2004). In addition, high environmental trace metals levels correlate with reproduction impairments (e.g. higher nest desertion, hatching failure and mortality; Eeva and Lehikoinen 1996; Sens et al. 2003; Eeva et al. 2009), and oxydative damages (Berglund et al. 2007), in passerines. However, the separate and interactive effects of a chronic exposure to trace metals are poorly known and previous correlative studies cannot exclude confounding urban factors also known to impair birds' reproduction (Halfwerk et al. 2011; Dominoni et al. 2013). While trace metals' effects on condition and reproductive success may greatly modulate birds' fitness and population dynamics, to the best of our knowledge, no experimental study demonstrated such effects.

Because of their toxicity, trace metals may select for detoxification mechanisms such as higher oxidative stress resistance or higher elimination rate of ingested metals. Interestingly, highly melanic plumage may be adapted to environments polluted with trace metals, both through direct and indirect effects of melanogenesis. First, high concentrations of metals are measured in feathers (Agusa et al. 2005), in which they are sequestrated, then eliminated during moulting. Therefore, metal transfer from the bloodstream into the feathers during feather growth (Burger 1993) could be an efficient detoxification mechanism. Metal linkage into feathers would be partly due to the negatively-charged free carboxyl, hydroxyl and amine functions of melanin, known to bind metal ions in vitro (Larsson and Tjälve 1978; Liu et al. 2004; Bridelli and Crippa 2007). Consequently, metal chelation is suggested as one of the main biological functions of melanin (McGraw 2003; Hong and Simon 2007; Chatelain et al. 2014). Previous studies demonstrated a positive correlation between some metal concentrations and plumage melanin-based colouration (Gochfeld et al. 1991; Niecke et al. 1999; Niecke et al. 2003; Chatelain et al. 2014) which may suggest that more melanic feathers would be able to store higher amounts of metals. Consequently, highly melanic birds would have a better ability to detoxify themselves

than paler birds by lowering their circulating metal burden. Although this detoxification mechanism could represent a significant driver of melanin-based plumage colouration polymorphism maintenance, it has been poorly investigated. Only some metals concentrations have been shown to be positively correlated to melanin-based plumage colouration (zinc, calcium and manganese; Niecke et al. 1999; Zduniak et al. 2014), while no such link has been demonstrated for highly toxic metals such as lead and cadmium, maybe because of the correlative nature of the studies (Gochfeld et al. 1991; Chatelain et al. 2014). Then, to the best of our knowledge, no study compared metal feather concentrations between differently melanin-coloured birds in controlled environmental conditions (ie. under the same metal exposure). Second, both the pleiotropic effect of the gene coding for melanin synthesis (POMC) and its linkage disequilibrium with various loci result in correlation between variation in melanin-based plumage colouration and several biological traits, including immunity, antioxidant capacity and stress resistance (Ducrest et al. 2008; Mckinnon and Pierotti 2010). Indeed, darker pigeons exhibited both a lower endoparasite intensity and a greater cellular immune response than paler ones (Jacquin et al. 2011). Moreover, eumelanin level in the barn owl was positively correlated with resistance to oxidative stress (Ducrest et al. 2008; Roulin et al. 2011) and to physiological stress (corticosterone synthesis; Almasi et al. 2010; Almasi et al. 2012). Therefore, melanin-based plumage colouration may shape birds' tolerance to trace metals.

Both direct (metals binding) and indirect (resistance to parasites, oxidative stress and physiological stress) associations between biological traits and plumage melanism may favour darker birds in environments polluted with trace metals. Accordingly, previous studies observed a higher frequency of darker feral pigeons in European cities (Obukhova 2007; Jacquin et al. 2013b), where environmental concentrations of metals are the highest (Azimi et al. 2005; Scheifler et al. 2006; Roux and Marra 2007; Kekkonen et al. 2012). However, there is no experimental evidence for fitness advantages of being more melanic in habitats polluted with trace metals.

Partly due to large emissions by anthropogenic activities, lead is the most abundant toxic metal in the environment (Azimi et al. 2005; Roux and Marra 2007). Although lead used in gasoline drastically diminished since the 70s (Jarup 2003), it remains of high ecological importance due to its accumulation into the soil (Roux and Marra 2007) and to the negative biological effects of a chronic exposure, even at low levels (Patrick 2006). In addition, zinc is the most abundant metal in the environment (Azimi et al. 2005). While being an essential nutrient (Prasad 1998; Prasad 2009), it induces harmful effects at high concentrations (Greenberg and Briemberg 2004; Bozym et al. 2010). Therefore, lead and zinc are likely to induce the strongest effects on

urban wildlife. For this reason, we chronically exposed feral pigeons (*Columba livia*), a highly polymorphic bird species with respect to its melanin-based plumage colouration, to lead and/or zinc in experimentally controlled concentrations inferred from previous measures in urban areas. First, for an ecotoxicological purpose, we investigated the effects of such exposures on lead and zinc concentrations in birds' feathers and blood, and on their condition and reproductive parameters. Then, we investigated whether the ability of feathers to store zinc and lead depends on their melanin-based colouration and, as a consequence, whether melanic birds maintain lower blood lead concentrations; because zinc blood concentrations are under strict homeostatic regulation (Maret 2008), no relationship was expected between plumage colouration and zinc blood levels. Finally, we tested whether melanin-based plumage colouration could be advantageous in metal polluted environments by investigating the interaction between plumage colouration and metal exposure on birds' condition and reproductive parameters.

METHODS

Subjects and Housing

Ninety six (48 males and 48 females genetically sexed) free-living adult feral pigeons (*Columba livia*) exhibiting various melanin-based plumage colourations were caught during winter 2013 (February/March) in several flocks within the Parisian agglomeration. The birds were immediately transferred in 8 outdoor aviaries (3.10 m x 2.00 m x 2.40 m) at the CEREEP field station (Centre d'Ecologie Expérimentale et Prédictive-Ecotron Ile-de-France, UMS 3194, Ecole Normale Supérieure, Saint-Pierre-lès-Nemours, France). Birds were fed *ad libitum* with a mix of maize, wheat and peas. The aviaries were enriched with a bowl of water used for bathing and with branches as perches. Birds were individually identified with a numbered plastic ring. At the end of the experiment, all birds were released back to the wild at their site of capture.

Plumage colouration measurement

Melanin is the most widespread pigment in the animal reign. It exists in two types: eumelanin and pheomelanin, responsible for the black and reddish colour of teguments respectively. Melanin-based plumage colouration in feral pigeons is highly heritable (Jacquin et al. 2013a). At their capture, birds were first categorised as eumelanic (grey to black pigmented) or pheomelanic (red pigmented), which defines what we called their melanin type. Then, eumelanic birds were individually photographed to precisely quantify their eumelanin level. Eumelanin

level was calculated as the percentage of black on the wing surface of birds (number of black pixels/number of white pixels x 100) using the Gimp image retouching and editing software, which is a reliable and repeatable estimation of melanin concentration (Jacquin et al. 2011). Fledglings born during the experiment were also photographed to assess their eumelanin level. Because of the small amount of pheomelanic birds (14 out of 96), the measure of a pheomelanin level was not relevant; we however estimated it to ensure that the percentage of pigmented surface did not significantly differ between eumelanic and pheomelanic birds ($F_{1,94}$=0.27, P=0.606).

Treatments

Two weeks before the start of the experiment, the birds were distributed in the aviaries in order to equilibrate both gender (6 females and 6 males per aviaries) and plumage colouration ($F_{1,94}$<0.01, P=0.974). However, because of their lower number (n=14), pheomelanic individuals were spared in only 6 aviaries. There was no confounding effect between flock and aviary (Chi^2=71.09, df=70, P=0.441). The aviaries were randomly assigned to one of the 4 following metal exposure treatments: exposed to lead only (*lead* group; 1ppm lead acetate, Sigma-Aldrich), exposed to zinc only (*zinc* group; 10ppm zinc sulphate, Prolabo), exposed to both lead and zinc (*lead+zinc* group; 1ppm lead acetate and 10ppm zinc sulphate) or control (*control* group; tap water with none added-metal). This resulted in 2 aviaries with 12 pigeons each (24 pigeons in total) per treatment. We chose these concentrations based on both lead blood concentrations measured in urban birds (ranging from 0,053 to 0,264ppm; (Roux and Marra 2007) and the gastrointestinal absorption rate of lead in zebra finches (<10%) calculated from (Dauwe, L. Bervoets, R. Blust, M. Ee 2002). Metals were diluted in tap water. Drinking troughs and baths were filled with the corresponding treated water every other day.

Scaled mass index

From the start to the 20th week of the experiment, all birds were captured once a week to be weighed to the nearest gram with a Pesola Newton scale. Scaled mass index was calculated according to the method described by Peig and Green (Peig and Green 2009; Peig and Green 2010).

Reproductive success measurements

Breeding success - A week after the start of the treatments six nest boxes per aviary were opened (a box per couple) to allow birds to mate and breed. A bird was considered as reproducing when it had laid or incubated at least one egg during the breeding season. Overall, 52 pigeons (28 females and 24 males) succeeded in reproduction.

Egg's quality measurement - Feral pigeons commonly produce two eggs clutches, one to 6 times a year. The day it had been laid, the egg was removed from the nest, weighed and measured (3 measures of eggs' maximum length and maximum width were taken, and then averaged). Egg volume was calculated as $V = 0.4866 \times Length \times (Width^2)$ (Paillisson et al. 2007). Eggs of the first, third and fifth clutches were put back in the nest to allow incubation (n=83) whereas the others were frozen (n=65). Shell, albumen and yolk of frozen eggs were separated, then weighed to the nearest µg (eggshells were previously oven-dried). Dried shell thickness was measured to the nearest µm with a specimeter (Mitutoya 0-1mm).

Hatching success - The hatch was successful when the chick was completely cleared from its shell and alive. 52 eggs successfully hatched.

Nestlings' growth measurement - The day it hatched, the chick was weighed and measured (3 measures of tarsus and wing length were taken, and then averaged; n=52). Weight, tarsus and wing measures were reiterated every day until 25 days old (note that nestlings' body mass slow down at 16 days old on average; Johnston and Janiga 1995). Growth (body mass, tarsus and wing length) was calculated as $W = \frac{A}{1+\exp(-k(t-t_i))}$, where W=body mass/tarsus length/wing length, A=asymptote (final body mass/tarsus length/wing length at the end of growth), k=growth rate constant t=age and t_i =the inflexion point of the curve (Newbrey and Reed 2009; Jacquin et al. 2012). Therefore we characterized nestlings' growth by its growth rate (k) and its age of slowing growth (t_i). Only the growth of nestlings which successfully fledged was calculated (n=41). Three months after the birds stopped growing, their weight, tarsus length and wing length were measured to assess their scaled mass index (n=40).

Fledging success – Nestling successfully fledged when it was found out the parental nest and was able to fly and to feed by itself.

Juveniles' condition – Three days after hatching, we measured juveniles' haematocrit, corresponding to the erythrocyte volume fraction of a blood sample (n=40). It is expected to be an indicator of general health status (Cooper 1975; Averbeck 1992). In addition, the number of leukocytes per 10000 erythrocytes was counted from blood smear. Slides were fixed with methanol during 5 minutes and coloured with GIEMSA (diluted 1:20) during 45 minutes. We

identified heterophils, eosinophils, lymphocytes and monocytes. Because glucocorticoid level decreases the number of circulating lymphocytes while it stimulates the influx of heterophils from the bone marrow, leukocyte profiles are suitable for identifying some physiological stress (Davis et al. 2008). Therefore, we calculated the heterophil/lymphocyte ratio. We also considered the total number of white blood cells (total number of leukocytes per 10000 erythrocytes) that is suggested to be an indication of birds' immunity (Davis et al. 2008).

Metals quantitative analyses

In blood – 10 weeks after the start of the experiment, 50µl of blood were collected from the brachial vein of each 96 adult pigeons and were immediately frozen until analyzes. Blood was defrosted and vortexed. Then, 200mg (±0.1 mg) were digested with 1ml HNO_3 solution (68%) during 24h at 80°C.

In feathers – 13 weeks after the start of the experiment, a secondary remige (the 5th) was removed a first time. Once the regrown feather finished its development and was devascularizated, it was plucked off and conserved in an individual plastic bag. Feathers were washed vigorously with 0.25M NaOH solution, rinsed energetically 3 times in ultrapure water (Milli-Q purified) to remove external contamination (Scheifler et al. 2006; Frantz et al. 2012), left 1h in ultrapure water and dried 12h at 80°C to dry mass. Barbs were removed from the rachis, weighed to the nearest 0.1 mg and digested following the method described above.

The product of digestion was transferred into plastic tubes and water was added to reach a final volume of 8ml; then, each sample was diluted by 2.5. Total lead and zinc concentrations were determined in all of the feather samples and 48 blood samples (6 females and 6 males amongst each four treatments) by mass spectrometry (quadrupole ICP-MS, XSerie II) and optical emission spectrometry (ICP-OES, JY 2000) respectively.

Statistical analyses

To distinguish the effects of lead and/or zinc exposure, we binary coded (absence/presence) the exposure to lead on one hand and the exposure to zinc on the other (table 1). First, we tested for correlations between the explanatory variables considered (lead exposure, zinc exposure, plumage melanin type and plumage eumelanin level among adults and among juveniles separately). Lead exposure significantly affected juveniles plumage eumelanin level ($F_{3,30}$=6.69, P=0.015; figure 1), with eumelanin level being higher among juveniles exposed to lead (*lead* and *lead+zinc* groups). Therefore, we were not able to include metals exposures and juveniles'

eumelanin level in the same model (see below). There was no significant relation between adults' eumelanin level and metals exposures among all adults, among adults that succeeded to breed, to give hatchlings and to give fledglings.

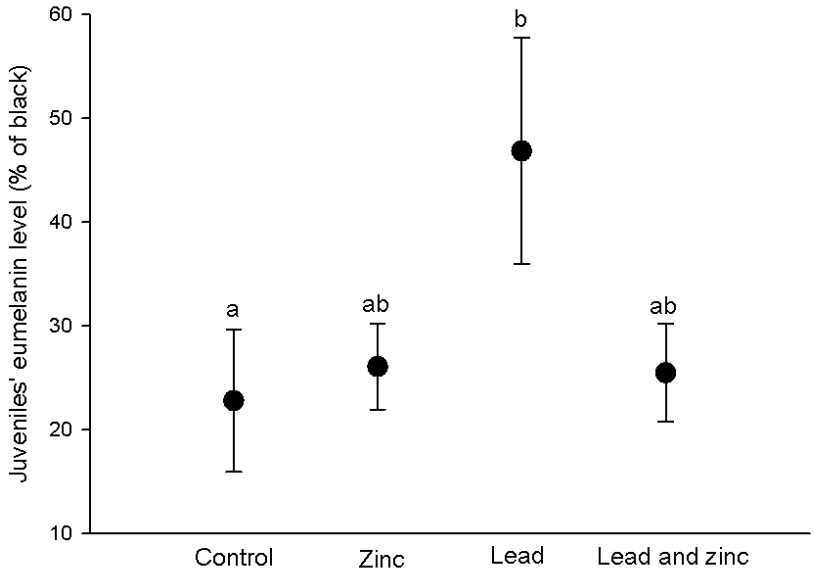


Fig. 1 Eumelanin level (% of black) of juveniles according to their metal exposure treatment.

For each dependant variable to be tested, we performed three successive models:

1) The ecotoxicological effect of lead and/or zinc exposure - in all birds (pheomelanic and eumelanic), we investigated the effects of lead and zinc exposure (birds' plumage colouration was not added in the model): lead exposure, zinc exposure and their interactions were the explanatory variables.

2) The effect of melanin type - in all birds, we investigated the effect of pigeons' melanin type (pheomelanic vs. eumelanic) in interaction with lead and zinc exposure: lead exposure, zinc exposure, melanin type and their interactions were the explanatory variables. Note that the interaction between zinc exposure and lead exposure was not tested because no pheomelanic birds have been exposed to both zinc and lead.

3) The effect of eumelanin level - in eumelanic birds, we investigated the effects of plumage eumelanin level in interaction with lead and zinc exposure: lead exposure, zinc exposure, eumelanin level and their interactions were the explanatory variables.

First, we investigated metals feathers concentrations using mixed linear models. Age was added into the models when testing the effects of metals alone (model 1) and of melanin type (model 2). However, there were too few pheomelanic juveniles (4) to test for the interaction between melanin type and age. When testing for the effects of eumelanin level (model 3) and because of colinearity issues (see above) that did not allow us to test the effects of metals exposure and eumelanin levels in the same model, we performed separated models for adults and juveniles. In juveniles, we first tested for the effect of eumelanin level alone; when significant, we then tested for the effect of lead exposure, zinc exposure and their interaction, and compared, when possible, the two models using the AIC. Because lead detected in the feathers of birds that were not exposed to lead arise mostly from birds' natural environment, the potential link between plumage colouration and lead feather concentrations may be concealed due to different histories between birds. Thus, we performed additional models investigating lead feather concentrations according to plumage colouration in birds exposed to lead (*lead* and *lead+zinc* groups), both in adults and juveniles, which exhibited significantly higher lead feather concentrations (see below). Second, we investigated metals blood concentrations using mixed linear models. Third, we investigated fitness parameters; linear mixed models with time and its interactions with the other variables listed above were performed to explain adults' scaled mass index variation along time; the individual was added as a random effect. Finally, we performed mixed linear models (egg's quality, nestlings' growth and scaled mass index at 3 days of age) or general mixed linear models for binomial distribution (breeding success hatchling success, fledging success, juveniles' total white blood cell and heterophil/lymphocyte ratio) to test for the interactions between parental colourations and metal exposure on the reproductive parameters measured. Both mother and father plumage colouration (melanin type or eumelanin level) were considered. The nest identity was added as random effect. For juvenile growth, scaled mass index and physiological state, parents' plumage colouration was replaced by juveniles' own plumage colouration. Because of the small amount of pheomelanic juveniles (4 over 40), the effect of melanin type was not tested for these variables. When testing for the effects of juveniles' eumelanin level and to take colinearity issues into account (see above), we first tested for the effect of eumelanin level alone; if significant, we then tested for the effect of lead exposure, zinc exposure and their interaction, and compared, when possible, the two models using the AIC. In all performed models, aviary number was added as random effect. Statistical analyses were performed using R software (version 3.0.2) and models were selected using AIC.

RESULTS

Trace metals feather concentrations

In all pigeons (pheomelanic and eumelanic), zinc feather concentrations were higher among birds exposed to zinc (*zinc* and *lead+zinc* groups; $F_{1,104}=4.25$, $P=0.042$; table 1) and in juveniles ($F_{1,104}=6.64$, $P=0.011$; 91.36ppm±1.68 and 99.27ppm±2.28 in adults and in juveniles respectively). Moreover, lead feather concentrations were higher among birds exposed to lead (*lead* and *lead+zinc* groups) than among the other ones (*zinc* and *control* groups; $F_{1,105}=15.09$, $P<0.001$; table 1).

In all birds (pheomelanic and eumelanic), zinc feather concentrations were higher among eumelanic birds than among pheomelanic ones ($F_{1,104}=25.64$, $P<0.001$; 96.26 ppm±1.15 and 72.54 ppm±5.13 in eumelanic and pheomelanic birds respectively). Melanin type was not retained in the model fitted for lead feather concentrations (among all birds and among birds exposed to lead).

In eumelanic adults, zinc feather concentrations increased with eumelanin level ($F_{1,63}=11.21$, $P<0.001$; figure 2). In eumelanic adults exposed to lead (*lead* and *lead+zinc* groups), lead feather concentrations increased with plumage eumelanin level ($F_{1,33}=5.12$, $P=0.030$; figure 3). In eumelanic juveniles, eumelanin level was not retained for the models fitted zinc feathers concentrations and for lead feathers concentrations among all juveniles and juveniles exposed to lead.

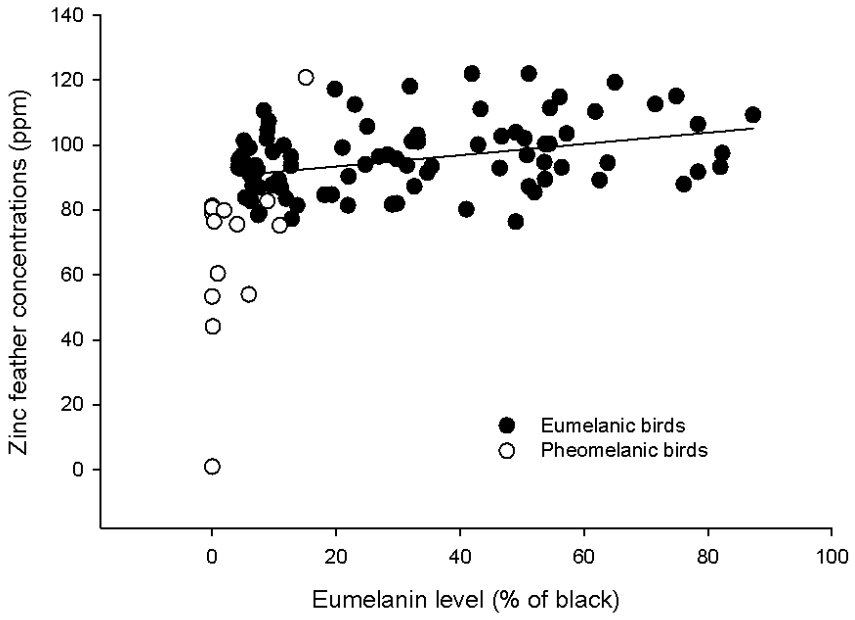


Fig. 2 Zinc feather concentrations (ppm) of adults according to their plumage.

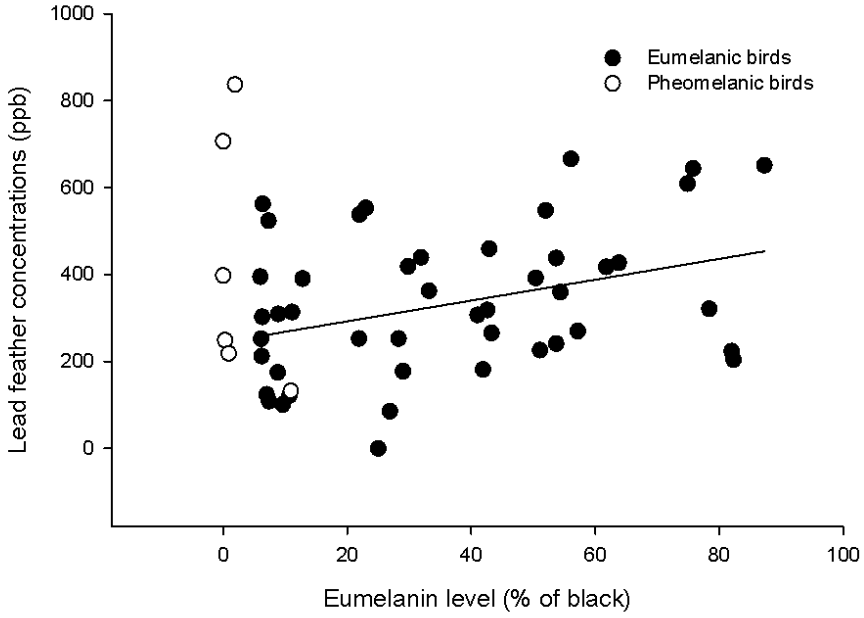


Fig. 3 Lead feather concentrations (ppb) of adults exposed to lead (*lead* and *lead+zinc* groups) according to their plumage eumelanin level.

Trace metals blood concentrations

In all adults (pheomelanic and eumelanic), lead concentrations in blood tended to depend on the interaction between zinc exposure and lead exposure ($F_{3,43}$=3.64, P=0.063; table 1): birds exposed to zinc only (*zinc* group) exhibited lower lead blood concentrations than birds exposed to both lead and zinc (*lead+zinc* group; $F_{1,21}$=10.79, P=0.004) and controls (*control* group; $F_{1,21}$=12.67, P=0.054). None of the considered variables were retained in the models fitted for zinc blood concentrations.

Scaled mass index variation

In all adults (pheomelanic and eumelanic), scaled mass index depended on the interaction between time, zinc exposure and lead exposure ($F_{1,96}$=8.93, P=0.003): scaled mass index decreased along time in *lead* ($F_{1,24}$=52.39, P<0.001), *control* ($F_{1,24}$=28.11, P<0.001) and *lead+zinc* groups ($F_{1,24}$=57.92, P<0.001) while time was not retained in the model fitted for scaled mass index in *zinc* group.

In eumelanic adults (pheomelanic and eumelanic), scaled mass index depended on the interaction between time, zinc exposure, lead exposure and eumelanin level ($F_{1,82}$=19.29, P<0.001; figure 4): scaled mass index decreased along time in *control* ($F_{1,20}$=35.64, P<0.001) and *lead+zinc*

group ($F_{1,24}$=57.92, P<0.001). In *lead* group, scaled mass index depended on the interaction between time and eumelanin level ($F_{1,19}$=40.02, P<0.001), with scaled mass index decreasing with time among the darkest birds only ($F_{1,9}$=87.10, P<0.001). Neither time nor eumelanic level was retained in the model fitted for scaled mass index among *zinc* group.

Melanin type was not retained in the model fitted for scaled mass index.

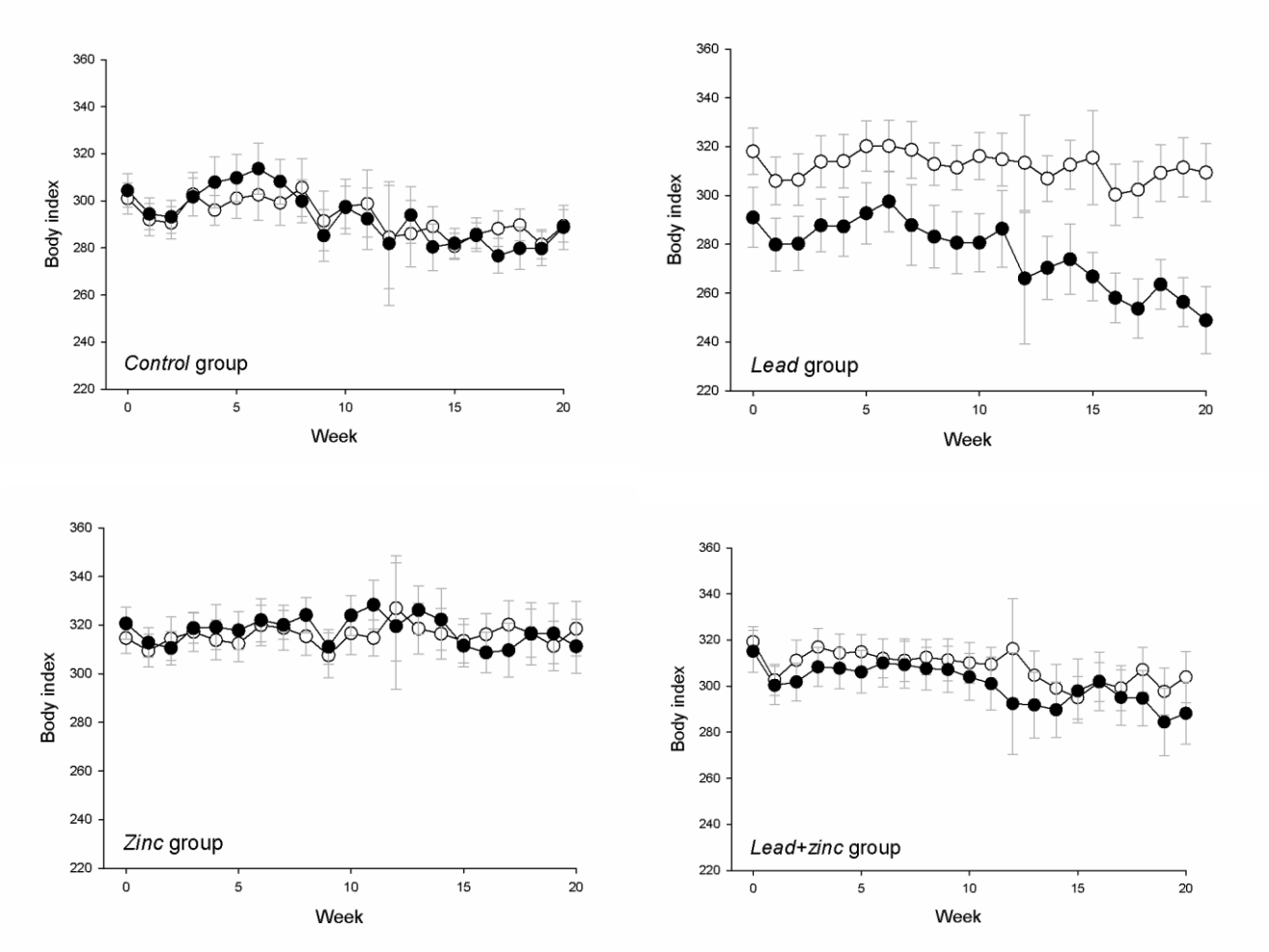


Fig. 4 Body index (mean± se; g) of adults from the start to the end of the experiment according to their exposure to lead and zinc and to their eumelanin level (in white when eumelanin level < 29 and in black when eumelanin level > 29).

Reproductive success

None of the considered variables were retained in the model fitted for birds' breeding success.

In all parents (pheomelanic and eumelanic), metal exposure was not retained in the models fitted for eggs' weight and volume, nor albumen and eggshell weight. However, yolk weight was higher in eggs from parents exposed to zinc (*zinc* and *lead+zinc* groups) than from the others

(*control* and *lead* groups; $F_{1,53}$=7.36, P=0.007; mean±se 4.22g±0.08 and 3.89g±0.09 respectively; table 1). Moreover, eggshell was thicker in eggs from parents exposed to zinc (*zinc* and *lead+zinc* groups; $F_{1,62}$=5.18, P=0.023; mean±se 0.49mm±0.01 and 0.47mm±0.00 respectively; table 1) while it was thinner in eggs from parents exposed to lead (*lead* and *lead+zinc* groups; $F_{1,62}$=8.24, P=0.004; mean±se 0.47mm±0.00 and 0.49mm±0.01 respectively; table 1).

None of the considered variables were retained in the model fitted for hatching success.

In all parents, nestlings exposed to lead (*lead* and *lead+zinc* groups) were significantly lighter than the other ones (*control* and *zinc* groups; $F_{1,52}$=4.17, P=0.041; mean±se 14.94g±0.72 and 17.20g±0.67 respectively; table 1). Zinc and lead exposure was not retained in the models fitted for tarsus and wing length. None of the considered variables were retained in the models fitted for one-day-old chick tarsus and wing length. With regard to nestlings' growth, none of the considered variables were retained in the models fitted for weight, tarsus and wing growth rate. However, the age at which weight and tarsus growth slowed down depended on the interaction between lead and zinc exposure ($F_{1,41}$=8.05, P=0.005; and $F_{1,41}$=9.66, P=0.002; figure 5). Indeed, growth slowed down earlier among juveniles exposed to lead only (*lead* group) than among juveniles exposed to both lead and zinc (*lead+zinc* group; $F_{1,24}$=5.53, P=0.019 and $F_{1,24}$=6.01, P=0.014 respectively) or in controls (*control* group; $F_{1,25}$=11.46, P=0.002 and $F_{1,25}$=19.52, P<0.001 respectively).

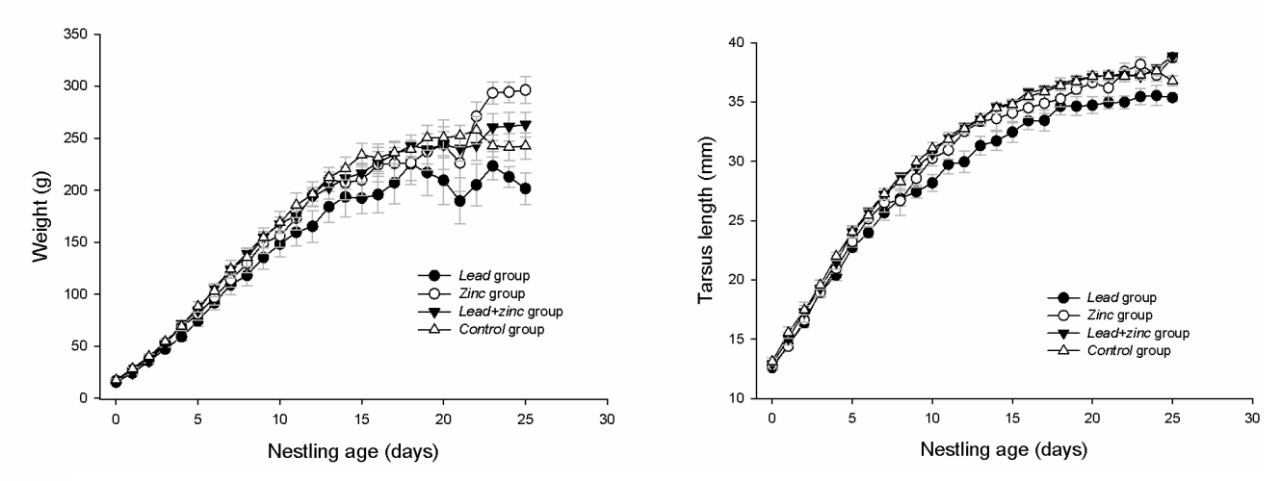


Fig. 5 Weight and tarsus growth of juveniles according to their exposure to lead and zinc

Zinc and lead exposure was not retained in the models fitted for the age at which wing growth slowed down and melanin type and eumelanin levels were retained in none of the models fitted for nestlings' growth. In all parents, fledging success was lower among pheomelanic fathers ($F_{1,59}$=8.13, P=0.004) and tended to be also lower among birds exposed to lead (*lead* and

lead+zinc groups; $F_{1,59}$=3.62, P=0.057). Eumelanin level was not retained in the model fitted for fledging success.

In all juveniles, scaled index was smaller in lead groups (*lead* and *lead+zinc* groups) than in the other groups (*control* and *zinc* groups; $F_{1,40}$=6.43, P=0.011; mean±se 293.91±19.11 and 349.73±20.02 respectively; table 1). Moreover, the number of white blood cells was higher in zinc groups (*zinc* and *lead+zinc* groups) than in the other groups ($F_{1,40}$=5.14, P=0.023; 6.0%±0.5 and 4.8%±0.3 respectively; table 1). The number of heterophils among the number of lymphocytes depended on the interaction between lead and zinc exposure ($F_{1,37}$=5.79, P=0.016; table 1): it was higher in *lead* group than in *control* group ($F_{1,20}$=4.65, P=0.031) and *lead+zinc* group ($F_{1,19}$=4.01, P=0.046). In eumelanic juveniles, eumelanin level was not retained in the model fitted for scaled mass index, the number of white blood cells and for the number of heterophils among the number of lymphocytes.

In all juveniles, scaled index was smaller in lead groups (*lead* and *lead+zinc* groups) than in the other groups (*control* and *zinc* groups; Chi^2=6.43, df=39, P=0.011; mean±se 293.91±19.11 and 349.73±20.02 respectively; table 1). Juveniles exposed to zinc (*zinc* group) had a higher scaled index in comparison to the others (*control* and *lead* groups; Chi^2=5.14, df=39, P=0.023; mean±se 347.90±19.52 and 300.49±19.93 respectively). Moreover, the number of white blood cells was higher in zinc groups (*zinc* and *lead+zinc* groups) than in the other groups (Chi^2=4.49, df=37, P=0.036; 6.0%±0.5 and 4.8%±0.3 respectively; table 1). The number of heterophils among the number of lymphocytes depended on the interaction between lead and zinc exposure (Chi^2=5.79, df=37, P=0.016; table 1): it was higher in *lead* group than in *control* group (Chi^2=4.65, df=20, P=0.031) and *lead+zinc* group (Chi^2=4.01, df=19, P=0.046). In eumelanic juveniles, eumelanin level was not retained in the model fitted for scaled mass index, the number of white blood cells and for the number of heterophils among the number of lymphocytes.

		Control	*Lead*	*Zinc*	*Lead+zinc*
Zinc concentrations (ppm)	Feather	91.91±2.22 $^{(a)}$	89.94±3.03 $^{(ab)}$	93.53±3.96 $^{(a)}$	99.24±2.07 $^{(b)}$
	Blood	3.96±0.17 $^{(a)}$	4.78±0.45 $^{(ab)}$	4.24±0.12 $^{(a)}$	5.57±0.22 $^{(b)}$
Lead concentrations (ppb)	Feather	249.53±51.07 $^{(a)}$	360.62±38.07 $^{(c)}$	170.11±25.00 $^{(b)}$	410.17±50.86 $^{(c)}$
	Blood	48.13±10.54 $^{(a)}$	50.95±9.46 $^{(a)}$	22.21±6.52 $^{(b)}$	60.02±9.24 $^{(a)}$
Scaled mass index variation		-0.79±0.34 $^{(a)}$	-1.22±0.38 $^{(a)}$	-0.25±0.26 $^{(b)}$	-0.93±0.29 $^{(a)}$
Proportion of breeders		0.625 $^{(a)}$	0.625 $^{(a)}$	0.542 $^{(a)}$	0.458 $^{(a)}$
Eggs' quality	Egg weight (g)	17.47±0.32 $^{(a)}$	17.40±0.30 $^{(a)}$	17.48±0.27 $^{(a)}$	17.80±0.26 $^{(a)}$
	Egg volume (dm^3)	15.63±0.28 $^{(a)}$	15.48±0.27 $^{(a)}$	15.60±0.25 $^{(a)}$	16.02±0.25 $^{(a)}$
	Yolk weight (g)	3.97±0.11 $^{(ab)}$	3.76±0.13 $^{(a)}$	4.20±0.12 $^{(b)}$	4.24±0.11 $^{(b)}$
	Albumen weight (g)	11.41±0.51 $^{(ab)}$	10.24±0.67 $^{(a)}$	11.06±0.44 $^{(ab)}$	11.64±0.29 $^{(b)}$
	Eggshell weight (g)	1.58±0.07 $^{(a)}$	1.41±0.10 $^{(a)}$	1.48±0.08 $^{(a)}$	1.61±0.08 $^{(a)}$

	Eggshell thickness (mm)	0.48±0.00 (a)	0.46±0.01 (b)	0.50±0.01 (a)	0.48±0.01 (ab)
Hatching success		0.708 (a)	0.680 (a)	0.667 (a)	0.789 (a)
One-day-old chick size	Weight (g)	17.76±0.092 (a)	14.75±0.83 (a)	16.77±0.95 (a)	15.27±0.96 (a)
	Tarsus length (mm)	13.21±0.26 (a)	12.47±0.16 (a)	12.74±0.30 (a)	12.90±0.29 (a)
	Wing length (mm)	14.43±0.28 (a)	13.67±0.29 (a)	14.00±0.33 (a)	13.98±0.36 (a)
Nestlings' growth	k weight	0.30±0.02 (a)	0.30±0.03 (a)	0.33±0.03 (a)	0.31±0.02 (a)
	t_i weight	16.09±0.52 (a)	12.95±0.93 (b)	14.90±1.95 (ab)	16.16±0.75 (a)
	k tarsus	0.21±0.01 (a)	0.19±0.02 (a)	0.22±0.01 (a)	0.21±0.01 (a)
	t_i tarsus	21.07±0.44 (a)	17.62±0.85 (b)	19.03±1.04 (ab)	20.97±0.87 (a)
	k wing	0.25±0.02 (a)	0.21±0.01 (a)	0.23±0.02 (a)	0.23±0.02 (a)
	t_i wing	6.51±0.54 (a)	7.34±0.79 (a)	8.02±1.28 (a)	7.25±1.43 (a)

Fledging success		0.882 *(ab)*	0.588 *(a)*	0.900 *(ab)*	0.933 *(b)*
Juveniles' condition at three months of age	Scaled mass index	343.46±26.36 *(a)*	238.41±15.05 *(b)*	361.37±31.80 *(a)*	339.33±25.59 *(a)*
	White blood cells ratio	0.005±0.003 *(a)*	0.005±0.005 *(a)*	0.007±0.001 *(a)*	0.006±0.000 *(a)*
	H/L ratio	0.031±0.014 *(a)*	0.185±0.094 *(b)*	0.077±0.038 *(ab)*	0.045±0.014 *(ab)*

Tab. 1 The measures of each investigated variable (mean±se) among the four treatment groups. We performed tests for each pair of treatment group. Letters were added to highlight the significant differences among treatments. k = the growth rate constant of the growth curve; t_i = the inflexion point of the growth curve. For juveniles white blood cell ratio, the test failed to detect any difference between the groups while birds exposed to zinc (zinc and lead+zinc groups) had a higher white blood cells ration than the other groups (lead and control groups). For scaled mass index, we wrote the slope of the regression between scaled mass index and time.

DISCUSSION

Our first aim was to investigate the effects of a chronic exposure to trace metals in concentrations encountered in urban areas on condition and reproductive parameters in feral pigeons. We expected trace metals experimentally provided to pigeons to be ingested and their concentrations to increase in feathers and blood. Accordingly, zinc and lead-exposure increased zinc and lead feathers concentrations respectively. Zinc and lead measured in the feathers were respectively 80 and 1.5 times less concentrated than the ones measured in feathers of urban pigeons (Nam et al. 2004; Adout et al. 2007; Hoff Brait and Antoniosi Filho 2011; Frantz et al. 2012; Chatelain et al. 2014), suggesting that our experimental exposure corresponds to the lower range of urban exposure. Interestingly, the increased feather concentrations in exposed pigeons open the possibility that feather renewal may be involved in metal burden regulation. Alternatively, metals exposures had no significant effects on zinc and lead blood concentrations. Other studies pointed out the irrelevance of metals blood concentrations to estimate recent exposures to trace metals. Indeed, metals blood concentration results from numerous mechanisms (lead clearance from bones, lead and zinc transfer into organs, bones and feathers (Cosson 1989; Gulson et al. 1996; Kim et al. 1998; Agusa et al. 2005). Moreover, in blood, we detected links between lead and zinc physiology regulations. Indeed, lead blood concentrations were lower among birds exposed to zinc only (*zinc* group) than among birds exposed to both lead and zinc (*zinc+lead* group); it could be due to the reduction of lead gastrointestinal absorption by zinc, as known in mammals (Cerklewski and Forbes 1976; El-Gazzar et al. 1978). These results point out the need for further studies investigating the physiological effects of metals cocktails, more representative of metal exposure in the wild.

The ingestion of zinc and lead significantly influenced birds' condition and reproductive success. Indeed, our study consistently demonstrated detrimental effects of lead exposure on these two variables. First, the scaled mass index decreased over the 20-week-long experiment among birds exposed to lead (*lead* and *lead+zinc* groups) while this index remained constant in *zinc* group and decreased less strongly in *control* group. Then, our results showed that lead-exposure decreased both eggshell thickness and juvenile growth, which is in accordance with a previous study in pied flycatcher (Eeva and Lehikoinen 1995). By diminishing eggshell thickness, lead may affect egg strength and therefore impair its resistance to impacts and its permeability (King and Robinson 1972). Lead affinity to calcium-binding sites (Simons 1993), would limit calcium deposition during eggshell formation and consequently decrease its thickness (Clunies et al.

1992). In addition, lead-exposure induced lighter one-day-old chicks. Because lead did not affect total, yolk nor albumen mass, it may not alter maternal investment in eggs but may be maternally transferred into the eggs and affect embryonic development (Burger 2002). Third, juveniles exposed to lead only (*lead* group) had a lower fledging success, possibly a consequence of a poorer condition at hatching (Grant 2008). Among fledglings, lead exposure also induced a shorter growth period of body mass and tarsus and a smaller scaled mass index at three months of age. It would be interesting to investigate whether the effect of lead on juveniles growth trajectories could impair their future survival or reproduction ("catch-up" hypothesis, see Criscuolo et al. 2008). Finally, juveniles exposed to lead only (*lead* group) had a higher number of heterophil/lymphocyte ratio, suggesting that they had a higher stress level (Davis et al. 2008).

In contrast, we observed beneficial effects of zinc on birds' scaled mass index and reproductive success. Birds exposed to zinc only (*zinc* group) maintained a constant scaled mass index along the experiment while this parameter generally decreased in birds of the other treatments. Zinc-exposure increased eggshell thickness (*zinc* and *lead+zinc* groups) and therefore may diminish the risk of egg-breaking (King and Robinson 1972). It also increased yolk mass, potentially increasing egg nutritive content (Noy and Sklan 1998). Accordingly, previous work reported a positive link between plasma zinc and vitellogenin production (Mitchell and Carlisle 1991). Since we did not find any difference in egg size and one-day-old chicks' size according to zinc exposure, this extra yolk mass may have been allocated to other physiological traits, such as immunity (see Li et al. 1998). Accordingly, juveniles exposed to zinc (*zinc* and *lead+zinc* groups) had a higher amount of white blood cells, an index of the immune system (Davis et al. 2008), than the other groups (*lead* and *control* groups). Finally, zinc-exposure had protective effects against lead: when provided along with zinc, lead did not negatively affect the length of nestlings' growth period, fledgling success nor stress level in juveniles. It also induced a non-significant protective effect on juveniles' scaled mass index. This protective effect may again result from zinc ability to reduce the absorption and retention of ingested lead (Cerklewski and Forbes 1976; El-Gazzar et al. 1978). The effects of trace metals observed on juveniles may be due to direct effects on juveniles or to indirect effects through parental investment. Further experimental studies are required to disentangle both effects.

In the wild, the toxic effects of trace metals on birds' condition and reproduction, such as the ones demonstrated here for lead, may select for a greater tolerance to trace metals, for instance through detoxification mechanisms or high antioxidant capacity. Because of the chelation properties of melanin, transfer of metals into the feathers would constitute an efficient mechanism

of blood detoxification. Moreover, plumage melanin level is linked to several biological functions, such as antioxidant capacity (Ducrest et al. 2008; Roulin et al. 2011), because of the pleiotropic gene coding for melanin synthesis (Ducrest et al. 2008). Both chelation properties of melanin and the pleiotropic effects linked to its synthesis may confer an advantage to birds exhibiting a more melanic plumage in environments with high levels of metals. Our second aim was thus to look for a relation between melanin-based plumage colouration and metals concentrations in blood and feathers and to investigate the interactive effects between metals exposures and plumage colouration on birds' condition and reproductive success.

First, in accordance with our previous work (Chatelain et al. 2014), we observed that zinc concentrations positively correlated with birds' plumage eumelanin level (ie. percentage of black). Interestingly, lead concentrations also increased with plumage eumelanin level in lead-exposed birds (*lead* and *lead+zinc* groups). To the best of our knowledge, our study is the first to observe a positive correlation between lead feather concentrations and plumage eumelanin level. Our results suggest that more melanic feathers would be able to store higher amounts of both zinc and lead. Therefore, melanin contained in the plumage could play a significant role in metals detoxification. However, we did not find the negative correlation between plumage eumelanin level and metals blood concentrations expected from our detoxification hypothesis. Nevertheless, metals blood concentration results depends on several mechanisms (Cosson 1989; Gulson et al. 1996; Kim et al. 1998; Agusa et al. 2005), which may hide such detoxification process.

Second, we did not find any predicted interaction between plumage eumelanin level and metals exposures on condition and reproductive parameters, except for the scaled mass index. In birds exposed to lead only (*lead* group), paler birds maintained their initial condition over the course of the experiment, while darker birds lost weight. Note however that darker birds had an initial lower scaled mass index than the paler ones, so that we cannot distinguish whether this result was due to the effect of initial scaled mass index or to eumelanin level. In the latter case, this result may suggest a disadvantage of a more melanic plumage in environments polluted with lead. Alternatively, it may also be the result of a trade-off between condition maintenance and other biological traits, such as parental investment, which would be in line with the higher survival rate of darker juveniles among birds exposed to lead only (*lead* group; see below).

Interestingly, plumage eumelanin level of three-month-old juveniles was higher in *lead* group than in the other groups. Because reproductive success (breeding, hatching and fledging success) did not depend on adults' eumelanin level alone or in interaction with metals exposures, this result may be due to higher survival rate of darker juveniles when exposed to lead only as compared to paler ones. This potential hypothesis is in accordance with the higher survival rate

of darker pigeon juveniles in a Parisian suburban environment (Récapet et al. 2013) and with the higher frequency of darker pigeons observed in European cities (Obukhova 2007). The synchronized growth of feathers in developing nestlings may exacerbate the transfer rate of lead into the plumage through melanin pigment, which would enhance detoxification in darker chicks and improve their survival. Alternatively, pleiotropic effects linking melanogenesis to other biological functions such as immunity and oxidative capacity (Ducrest et al. 2008) may cause a better ability of darker chicks to cope with the immunosuppressive (Snoeijs et al. 2004; Gasparini et al. 2014) and oxidative effects of lead. Indeed, darker pigeons exhibited both a lower endoparasite intensity and a greater cellular immune response than paler ones (Jacquin et al. 2011). Moreover, eumelanin level was positively correlated with resistance to oxidative stress in the barn owl (Ducrest et al. 2008; Roulin et al. 2011).

Finally, eumelanic birds had higher zinc feather concentrations than pheomelanic ones. It may be explained by a greater ability of eumelanin to bind zinc through its higher concentration of carboxylic acid groups (5,6-dihydroxyindole-2-carboxylic acid; DHICA; Hong and Simon 2007). Pheomelanic birds did not exhibit higher zinc blood concentrations, suggesting that pheomelanic birds' physiology may require higher amounts of zinc. For instance, we previously experimentally demonstrated that pheomelanic pigeons raise a stronger humoral immune response, known to be zinc dependent (Prasad 1998), than eumelanic pigeons (Chatelain et al. unpublished data). Moreover, pheomelanic plumage is associated with high oxidative damages in birds (Roulin et al. 2011; Galván et al. 2012) that may trigger the mobilization of significant amount of zinc by detoxification enzymes (Prasad 2009). In addition, fledging success was lower in juveniles reared by a pheomelanic father. This result suggests a reproductive cost of pheomelanin, which may explain the lower frequency of pheomelanic pigeons than the one of eumelanic pigeons (Johnston and Janiga 1995).

In our work, the chronic exposure to lead or zinc had respectively consistent negative and positive effects on pigeons' condition and reproductive success. Interestingly, these effects were obtained with concentrations much lower than commonly found in urban areas. Therefore, lead toxic effects are most probably even stronger in these habitats and may consequently deeply affect life history traits and urban birds' population dynamics. Our study suggests that zinc may partially compensate for lead noxious effects, though dose-dependent effects (Prasad 1998; Greenberg and Briemberg 2004; Prasad 2009; Bozym et al. 2010) stress the need for further investigating the effects of this metal in concentrations representative of urban areas. In the wild, trace metals,

when their detrimental effects are higher than their beneficial ones, may select for more tolerant phenotypes. High plumage eumelanin level, associated with a higher ability to store zinc and lead in feathers, may enable pigeons to better cope with trace metals and be consequently selected in urban areas. Furthermore, the higher survival rate of darker chicks exposed to lead suggests that lead may constitute a selective pressure and may be involved in melanin-based plumage colouration evolution. The differences in plumage colouration frequencies observed between urban and rural habitats (Obukhova 2007; Jacquin et al. 2013b) suggest a cost of harbouring a black plumage in areas not polluted with toxic metals, maybe through the sequestration in the plumage of useful metals.

More generally, trace metals likely exert selective pressures on polymorphic animal species with respect to their melanin-based inert skin appendage (plumage, fur, scale) colouration (e.g. house sparrows, great tits, eastern grey squirrels, house mice, vipers, moth, etc.). Nonetheless, melanin-based colouration is associated with numerous biological traits (e.g. thermoregulation, conspicuousness, immunity, personality, etc.); consequently, the various selective pressures exerted by trace metals exposure, predation or parasitism may have synergic or antagonist effects on the evolution of melanin-based inert skin appendage colouration and the result of such trade-offs may explain the temporal and spatial heterogeneity of melanin-based inert skin appendage colouration frequencies.

ACKNOWLEDGMENT

All experiments were carried out in strict accordance with the recommendations of the "European Convention for the Protection of vertebrate Animals used for Experimental and Other Scientific Purposes" and were conducted under the authorizations of the "Ministère de l'éducation nationale, de l'enseignement supérieur et de la recherche" (authorization N_00093.02) and the "Direction Départementale des Services Vétérinaires de Seine et-Marne" (authorization N_ 77-05). We thank the "Mairie de Paris" (Thomas Charachon) for allowing the capture of birds and the Centre de Recherche en Ecologie Expérimentale et Prédictive (CEREEP) which provided logistic support for the field work of this study. We are very thankful to S. Leclaire, T. Gayet, S. Pollet, S. Hasnaoui, F. Lorente, S. Perret and B. Decencière for their help all along the field work. We have no conflict of interest.

REFERENCES

Adout, A., D. Hawlena, R. Maman, O. Paz-Tal, and Z. Karpas. 2007. Determination of trace elements in pigeon and raven feathers by ICPMS. International Journal of Mass Spectrometry 267:109–116.

Agusa, T., T. Matsumoto, T. Ikemoto, Y. Anan, R. Kubota, G. Yasunaga, T. Kunito, et al. 2005. Body distribution of trace elements in black-tailed gulls from Rishiri Island, Japan: Age-dependent accumulation and transfer to feathers and eggs. Environmental Toxicology and Chemistry 24:2107–2120.

Almasi, B., L. Jenni, S. Jenni-Eiermann, and A. Roulin. 2010. Regulation of stress response is heritable and functionally linked to melanin-based coloration. Journal of Evolutionary Biology 23:987–996.

Almasi, B., A. Roulin, F. Korner-Nievergelt, S. Jenni-Eiermann, and L. Jenni. 2012. Coloration signals the ability to cope with elevated stress hormones: effects of corticosterone on growth of barn owls are associated with melanism: Plumage coloration and stress sensitivity. Journal of Evolutionary Biology 25:1189–1199.

Averbeck, C. 1992. Haematology and blood chemistry of healthy and clinically abnormal great black-backed gulls (Larus Marinus) and herring gulls (Larus Argentatus). Avian Pathology 21:215–223.

Azimi, S., V. Rocher, M. Muller, R. Moilleron, and D. R. Thevenot. 2005. Sources, distribution and variability of hydrocarbons and metals in atmospheric deposition in an urban area (Paris, France). Science of The Total Environment 337:223–239.

Berglund, A., J. Sturve, L. Forlin, and N. Nyholm. 2007. Oxidative stress in pied flycatcher (Ficedula hypoleuca) nestlings from metal contaminated environments in northern Sweden. Environmental Research 105:330–339.

Bozym, R. A., F. Chimienti, L. J. Giblin, G. W. Gross, I. Korichneva, Y. Li, S. Libert, et al. 2010. Free zinc ions outside a narrow concentration range are toxic to a variety of cells in vitro. Experimental Biology and Medicine 235:741–750.

Bridelli, M. G., and P. R. Crippa. 2007. Theoretical analysis of the adsorption of metal ions to the surface of melanin particles. Adsorption 14:101–109.

Burger, J. 1993. Metals in avian feathers: bioindicators of environmental pollution. Reviews of Environmental Contamination and Toxicology 5:203–311.

Burger, J. 2002. Food chain differences affect heavy metals in bird eggs in Barnegat Bay, New Jersey. Environmental Research 90:33–39.

Burger, J., and M. Gochfeld. 2004. Effects of lead and exercise on endurance and learning in young herring gulls. Ecotoxicology and Environmental Safety 57:136–144.

Cerklewski, F. L., and R. M. Forbes. 1976. Influence of dietary zinc on lead toxicity in the rat. The Journal of Nutrition 106:689–696.

Chatelain, M., J. Gasparini, L. Jacquin, and A. Frantz. 2014. The adaptive function of melanin-based plumage coloration to trace metals. Biology Letters 10:20140164–20140164.

Clunies, M., D. Parks, and S. Leeson. 1992. Calcium and phosphorus metabolism and eggshell thickness in laying hens producing thick or thin shells. Poultry Science 71:490–498.

Cooper, J. 1975. Haematological investigations in east american birds of prey 11:389–394.

Cosson, R. P. 1989. Relationships between heavy metal and metallothionein-like protein levels in the liver and kidney of two birds: The greater flamingo and the little egret. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology 94:243–248.

Criscuolo, F., P. Monaghan, L. Nasir, and N. B. Metcalfe. 2008. Early nutrition and phenotypic development: “catch-up” growth leads to elevated metabolic rate in adulthood. Proceedings of the Royal Society B: Biological Sciences 275:1565–1570.

Dauwe, L. Bervoets, R. Blust, M. Ee, T. 2002. Tissue levels of lead in experimentally exposed zebra finches (Taeniopygia guttata) with particular attention on the use of feathers as biomonitors. Archives of Environmental Contamination and Toxicology 42:88–92.

Davis, A. K., D. L. Maney, and J. C. Maerz. 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Functional Ecology 22:760–772.

Dominoni, D., M. Quetting, and J. Partecke. 2013. Artificial light at night advances avian reproductive physiology. Proceedings of the Royal Society B: Biological Sciences 280:20123017–20123017.

Ducrest, A., L. Keller, and A. Roulin. 2008. Pleiotropy in the melanocortin system, coloration and behavioural syndromes. Trends in Ecology & Evolution 23:502–510.

Eeva, T., M. Ahola, and E. Lehikoinen. 2009. Breeding performance of blue tits (Cyanistes caeruleus) and great tits (Parus major) in a heavy metal polluted area. Environmental Pollution 157:3126–3131.

Eeva, T., and E. Lehikoinen. 1995. Egg shell quality, clutch size and hatching success of the great tit (Parus major) and the pied flycatcher (Ficedula hypoleuca) in an air pollution gradient. Oecologia 102:312–323.

Eeva, T., and E. Lehikoinen. 1996. Growth and mortality of nestling great tits (Parus major) and pied flycatchers (Ficedula hypoleuca) in a heavy metal pollution gradient. Oecologia 108:631–639.

El-Gazzar, R. M., V. N. Finelli, J. Boiano, and H. G. Petering. 1978. Influence of dietary zinc on lead toxicity in rats. Toxicology Letters 1:227–234.

Frantz, A., M.-A. Pottier, B. Karimi, H. Corbel, E. Aubry, C. Haussy, J. Gasparini, et al. 2012. Contrasting levels of heavy metals in the feathers of urban pigeons from close habitats suggest limited movements at a restricted scale. Environmental Pollution 168:23–28.

Galván, I., J. Erritzøe, K. Wakamatsu, and A. P. Møller. 2012. High prevalence of cataracts in birds with pheomelanin-based colouration. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 162:259–264.

Gasparini, J., L. Jacquin, K. Laroucau, F. Vorimore, E. Aubry, M. Castrec-Rouëlle, and A. Frantz. 2014. Relationships between metals exposure and epidemiological parameters of two pathogens in urban pigeons. Bulletin of Environmental Contamination and Toxicology 92:208–212.

Gochfeld, M., J. Saliva, F. Lesser, T. Shukla, D. Bertrand, and J. Burger. 1991. Effects of color on cadmium and lead levels in avian contour feathers. Archives of environmental contamination and toxicology 20:523–526.

Grant, M. C. 2008. Relationships between egg size, chick size at hatching, and chick survival in the Whimbrel Numenius phaeopus. Ibis 133:127–133.

Greenberg, S. A., and H. R. Briemberg. 2004. A neurological and hematological syndrome associated with zinc excess and copper deficiency. Journal of Neurology 251:111–114.

Gulson, B., K. J. Mizon, M. J. Korsch, D. Howarth, A. Phillips, and J. Hall. 1996. Impact on blood lead in children and adults following relocation from their source of exposure and contribution of skeletal tissue to blood lead. Bulletin of Environmental Contamination and Toxicology 56:543–550.

Halfwerk, W., L. J. M. Holleman, Ck. M. Lessells, and H. Slabbekoorn. 2011. Negative impact of traffic noise on avian reproductive success: Traffic noise and avian reproductive success. Journal of Applied Ecology 48:210–219.

Hoff Brait, C. H., and N. R. Antoniosi Filho. 2011. Use of feathers of feral pigeons (Columba livia) as a technique for metal quantification and environmental monitoring. Environmental Monitoring and Assessment 179:457–467.

Hong, L., and J. D. Simon. 2007. Current understanding of the binding sites, capacity, affinity, and biological significance of metals in melanin. The Journal of Physical Chemistry B 111:7938–7947.

Hsu, M. J., K. Selvaraj, and G. Agoramoorthy. 2006. Taiwan's industrial heavy metal pollution threatens terrestrial biota. Environmental Pollution 143:327–334.

Jacquin, L., C. Haussy, C. Bertin, K. Laroucau, and J. Gasparini. 2013a. Darker female pigeons transmit more specific antibodies to their eggs than do paler ones. Biological Journal of the Linnean Society 108:647–657.

Jacquin, L., P. Lenouvel, C. Haussy, S. Ducatez, and J. Gasparini. 2011. Melanin-based coloration is related to parasite intensity and cellular immune response in an urban free living bird: the feral pigeon Columba livia. Journal of Avian Biology 42:11–15.

Jacquin, L., C. Recapet, P. Bouche, G. Leboucher, and J. Gasparini. 2012. Melanin-based coloration reflects alternative strategies to cope with food limitation in pigeons. Behavioral Ecology 23:907–915.

Jacquin, L., C. Récapet, A.-C. Prévot-Julliard, G. Leboucher, P. Lenouvel, N. Erin, H. Corbel, et al. 2013b. A potential role for parasites in the maintenance of color polymorphism in urban birds. Oecologia 173:1089–1099.

Jarup, L. 2003. Hazards of heavy metal contamination. British Medical Bulletin 68:167–182.

Johnston, R., and M. Janiga. 1995. Feral pigeons. Oxford University Press.

Kekkonen, J., I. K. Hanski, R. A. Väisänen, and J. E. Brommer. 2012. Levels of heavy metals in house Sparrows (Passer domesticus) from urban and rural habitats of southern Finland. Ornis Fennica 89.

Kim, E. Y., R. Goto, S. Tanabe, H. Tanaka, and R. Tatsukawa. 1998. Distribution of 14 elements in tissues and organs of oceanic seabirds. Archives of Environmental Contamination and Toxicology 35:638–645.

King, N. R., and D. S. Robinson. 1972. The use of the scanning electron microscope for comparing the structure of weak and strong egg shells. Journal of Microscopy 95:437–443.

Larsson, B., and H. Tjälve. 1978. Studies on the melanin-affinity of metal ions. Acta Physiologica Scandinavica 104:479–484.

Liu, Y., L. Hong, V. R. Kempf, K. Wakamatsu, S. Ito, and J. D. Simon. 2004. Ion-exchange and adsorption of Fe (III) by sepia melanin. Pigment Cell Research 17:262–269.

Li, X., T. Nakano, H. H. Sunwoo, B. H. Paek, H. S. Chae, and J. S. Sim. 1998. Effects of egg and yolk weights on yolk antibody (IgY) production in laying chickens. Poultry Science 77:266–270.

Maret, W. 2008. Metallothionein redox biology in the cytoprotective and cytotoxic functions of zinc☆. Experimental Gerontology 43:363–369.

McGraw, K. J. 2003. Melanins, metals, and mate quality. Oikos 402–406.

Mckinnon, J. S., and M. E. R. Pierotti. 2010. Colour polymorphism and correlated characters: genetic mechanisms and evolution. Molecular Ecology 19:5101–5125.

Mitchell, M., and A. Carlisle. 1991. Plasma zinc as an index of vitellogenin production and reproductive status in the domestic fowl. Comparative Biochemistry and Physiology Part A: Physiology 100:719–724.

Nam, D.-H., D.-P. Lee, and T.-H. Koo. 2004. Monitoring for lead pollution using feathers of feral pigeons (Columba livia) from Korea. Environmental Monitoring and Assessment 95:13–22.

Newbrey, J. L., and W. L. Reed. 2009. Growth of yellow-headed blackbird Xanthocephalus xanthocephalus nestlings in relation to maternal body condition, egg mass, and yolk carotenoids concentrations. Journal of Avian Biology 40:419–429.

Niecke, M., M. Heid, and A. Krüger. 1999. Correlations between melanin pigmentation and element concentration in feathers of white-tailed eagles (Haliaeetus albicilla). Journal für Ornithologie 140:355–362.

Niecke, M., S. Rothlaender, and A. Roulin. 2003. Why do melanin ornaments signal individual quality? Insights from metal element analysis of barn owl feathers. Oecologia 137:153–158.

Noy, Y., and D. Sklan. 1998. Yolk utilisation in the newly hatched poult. British Poultry Science 39:446–451.

Obukhova, N. 2007. Polymorphism and phene geography of the blue rock pigeon in Europe. Russ. J. Genet 43:492–501.

Paillisson, J.-M., S. Reeber, A. Carpentier, and L. Marion. 2007. Reproductive parameters in relation to food supply in the whiskered tern (Chlidonias hybrida). Journal of Ornithology 148:69–77.

Patrick, L. 2006. Lead toxicity, a review of the literature. Part 1: Exposure, evaluation, and treatment. Alternative Medicine Review: A Journal of Clinical Therapeutic 11:2–22.

Peig, J., and A. J. Green. 2009. New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. Oikos 118:1883–1891.

Peig, J., and A. J. Green. 2010. The paradigm of body condition: a critical reappraisal of current methods based on mass and length: The paradigm of body condition. Functional Ecology 24:1323–1332.

Prasad, A. S. 1998. Zinc and immunity. Molecular and cellular biochemistry 188:63–69.

Prasad, A. S. 2009. Zinc: role in immunity, oxidative stress and chronic inflammation: Current Opinion in Clinical Nutrition and Metabolic Care 12:646–652.

Récapet, C., L. Dauphin, L. Jacquin, J. Gasparini, and A.-C. Prévot-Julliard. 2013. Eumelanin-based colouration reflects local survival of juvenile feral pigeons in an urban pigeon house. Journal of Avian Biology 44:583–590.

Roulin, A., B. Almasi, K. S. Meichtry-Stier, and L. Jenni. 2011. Eumelanin- and pheomelanin-based colour advertise resistance to oxidative stress in opposite ways: Melanin colour and resistance to oxidative stress. Journal of Evolutionary Biology 24:2241–2247.

Roux, K. E., and P. P. Marra. 2007. The presence and impact of environmental lead in passerine birds along an urban to rural land use gradient. Archives of Environmental Contamination and Toxicology 53:261–268.

Scheifler, R., M. Cœurdassier, C. Morilhat, N. Bernard, B. Faivre, P. Flicoteaux, P. Giraudoux, et al. 2006. Lead concentrations in feathers and blood of common blackbirds (Turdus merula) and in earthworms inhabiting unpolluted and moderately polluted urban areas. Science of The Total Environment 371:197–205.

Sens, E. J., T. Dauwe, R. Pinxten, and M. Eens. 2003. Breeding performance of great tits (Parus major) along a gradient of heavy metal pollution. Environmental Toxicology and Chemistry 22:1140–1145.

Simons, T. J. 1993. Lead-calcium interactions in cellular lead toxicity. Neurotoxicology 14:77–85.

Snoeijs, T., T. Dauwe, R. Pinxten, V. Darras, L. Arckens, and M. Eens. 2005. The combined effect of lead exposure and high or low dietary calcium on health and immunocompetence in the zebra finch. Environmental Pollution 134:123–132.

Snoeijs, T., T. Dauwe, R. Pinxten, F. Vandesande, and M. Eens. 2004. Heavy metal exposure affects the humoral immune response in a free-living small songbird, the great tit (Parus major). Archives of Environmental Contamination and Toxicology 46.

Zduniak, P., A. Surmacki, K. Erciyas-Yavuz, M. Chudzińska, and D. Barałkiewicz. 2014. Are there different requirements for trace elements in eumelanin- and pheomelanin-based color production? A case study of two passerine species. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 175:96–10

Chapitre 3:

Métaux traces, mélanisme et immunité

Introduction

Des études précédentes suggèrent que l'exposition à certains métaux traces modifierait les réponses immunitaires des organismes. Par exemple, des mésanges charbonnières nichant proche d'une usine métallurgique présentent une plus faible production d'anticorps spécifiques (i.e. d'anticorps synthétisés en réponse à l'injection d'un antigène) que les individus nichant plus loin (Snoeijs et al., 2004). Chez le pigeon biset, l'intensité parasitaire est respectivement positivement et négativement corrélée aux concentrations en zinc et en plomb dans les plumes (Gasparini et al., 2014). De plus, chez l'Homme, l'administration de zinc augmente la taille du thymus et le nombre de lymphocytes T (Chandra and Dayton, 1982), alors que l'exposition au plomb est responsable de la production d'auto-anticorps et diminue l'activité des lymphocytes T (Mishra, 2009). En modifiant la sécrétion d'hormones de stress tels que les corticoïdes, les métaux traces peuvent également induire une immunosuppression (Baos et al., 2006; Cory-Slechta et al., 2004; Franceschini et al., 2009; Herring et al., 2012; Schulz et al., 2006; Vyskočil et al., 1990; Wayland, 2002). Aussi, les métaux traces présents dans l'environnement urbain sont susceptibles de modifier l'immunité des individus.

Par ailleurs, certaines études suggèrent un lien indirect entre la coloration mélanique du plumage et l'immunité, résultant des effets pléiotropes associés à la synthèse de la mélanine (Ducrest et al., 2008). Par exemple, les pigeons bisets au plumage le plus mélanique montrent une réponse inflammatoire plus forte, ainsi qu'une plus faible intensité en parasites sanguins par rapport aux pigeons les plus clairs (Jacquin et al., 2011). Aussi, le degré de mélanisme du plumage serait positivement associé à l'immunité, non seulement chez les individus exposés à des métaux traces toxiques comme le plomb - ceci grâce aux liens génétiques liant mélanisme, immunité, stress oxydatif et stress physiologique et au rôle détoxifiant de la mélanine du plumage - mais aussi chez les individus non exposés aux métaux traces, du fait encore une fois des liens génétiques entre mélanisme et immunité.

Méthodes

Lors de l'expérience réalisée au cours de l'année 2013, plusieurs paramètres de l'immunité ont été mesurés chez l'ensemble des adultes: l'immunité humorale primaire et secondaire (i.e. la production d'anticorps spécifiques suite à l'injection d'un antigène auquel les individus étaient

alors naïfs), l'immunité cellulaire (i.e. la réponse inflammatoire non spécifique suite à l'injection d'un antigène auquel les individus étaient également naïfs), la prévalence et l'intensité parasitaire (i.e. le nombre de parasites sanguins), ainsi que la capacité de lyse et d'hémagglutination.

Conclusion

Le plomb est connu pour avoir des effets immunosuppresseurs (Redig et al., 1991; Snoeijs et al., 2004; Youssef et al., 1996), alors que le zinc est un oligoélément essentiel à la mise en place de l'immunité (Prasad, 1998) et est toxique pour de nombreux microorganismes (Babich and Stotzky, 1978; Wirth et al., 1989). En accord avec ces études, nos résultats mettent en évidence qu'une exposition chronique à des concentrations faibles en plomb et en zinc engendre respectivement des effets négatifs et positifs sur l'immunité du pigeon biset. De plus, seuls certains paramètres immunitaires ont été affectés par les expositions au plomb et/ou au zinc (immunité humorale et cellulaire), suggérant que l'ensemble des paramètres constituant l'immunité ne covarient pas systématiquement face à un même facteur environnemental ; ceci souligne l'importance de réaliser des études intégratives, c'est-à-dire balayant le plus de paramètres de l'immunité possible afin de mieux comprendre les effets des métaux traces sur la capacité des individus à se défendre face à des infections. Pour finir, nos résultats montrent un effet antagoniste entre le plomb et le zinc, le zinc permettant de compenser certains effets négatifs liés à l'exposition au plomb. *In natura*, les individus sont exposés à un assemblage de métaux pouvant avoir des effets antagonistes, comme montrés dans notre étude, ou synergiques et il apparait essentiel de mieux cerner ces interactions afin d'estimer la réelle toxicité des métaux traces en milieu naturel et de comprendre leur rôle dans l'évolution des interactions hôtes-parasites.

Contrairement à notre hypothèse, nos résultats ne montrent pas de lien entre le degré d'eumélanisme du plumage et l'intensité des réponses immunitaires testées. Par contre, nous avons mis en évidence une différence significative entre l'immunité des individus eumélaniques et celle des individus phéomélaniques, ces derniers montant une réponse immunitaire humorale secondaire plus élevée. Par ailleurs, seule la réponse immunitaire cellulaire (i.e. la réponse inflammatoire) dépend d'une interaction entre le degré d'eumélanisme du plumage et l'exposition aux métaux traces : chez les individus exposés au plomb, la réponse immunitaire cellulaire diminue avec le degré d'eumélanisme du plumage. Cette relation est contraire à celle attendue sous l'hypothèse d'un rôle positif du mélanisme sur l'immunité et sur la détoxication. En effet, ce résultat suggère que les individus les plus mélaniques seraient davantage sensibles à une exposition chronique au plomb; bien que notre étude ne permette pas de déterminer le

mécanisme sous-jacent à cette différence de sensibilité, nous pouvons penser que, les individus mélaniques transférant davantage de plomb dans leurs plumes (voir Chapitre 2), ceux-ci maintiendraient des concentrations internes en plomb (i.e. dans les organes) plus faibles, et ainsi des concentrations sanguines en corticostérone immunosuppressive plus élevées.

D'un point de vue évolutif, il est difficile d'affirmer le rôle bénéfique de monter une réponse immunitaire forte. D'un côté, l'immunité est un paramètre physiologique semblant influencer fortement la survie des individus (Cichon and Dubiec, 2005; Hanssen et al., 2004; Møller and Saino, 2004) ; en effet, elle détermine la capacité des individus à faire face aux parasites (protozoaires, bactéries, virus, vers intestinaux, etc.), ainsi qu'à, chez les femelles des vertébrés, assurer l'immunité précoce de ses descendants alors immunonaïfs (i.e. sans défense immunitaire propre). D'un autre côté, une telle réponse immunitaire induit un coût énergétique non négligeable (Bonneaud et al., 2003; Hanssen et al., 2004). Par conséquent, les coûts et bénéfices associés à l'immunité sont susceptibles de dépendre de la probabilité d'infection, c'est-à-dire de l'abondance parasitaire dans l'environnement et de la densité de population, ainsi que de la virulence des parasites. Par exemple, lorsque la probabilité d'infection est élevée, comme c'est le cas en milieu urbain (Giraudeau et al., 2014), cela pourrait, chez les individus répondant fortement à l'infection, engendrer un coût important lié à la sur-stimulation du système immunitaire (Eraud et al., 2009; Hanssen et al., 2004; Lochmiller and Deerenberg, 2000). Aussi, la plus faible réponse immunitaire des individus eumélaniques d'une part, et des individus les plus mélaniques d'autre part, pourrait leur permettre d'économiser de l'énergie pour d'autre processus biologiques comme la détoxication, ce qui leur confèrerait un avantage sélectif dans un environnement où la probabilité d'infection est élevée, comme en milieu urbain. Bien qu'elles permettraient d'expliquer la rareté des individus phéomélaniques et la forte fréquence des pigeons biset foncés en milieu urbain, ces hypothèses restent spéculatives et soulignent l'importance d'approfondir nos connaissances sur les relations entre métaux traces, immunité et mélanisme du plumage, ainsi que sur le lien entre force de la réponse immunitaire et aptitude phénotypique en fonction des paramètres environnementaux comme l'abondance parasitaire.

Ce chapitre prend la forme d'un article soumis dans Ecotoxicology.

Trace metals, melanin-based pigmentation and their interaction shape immunity in feral pigeons (*Columba livia*)

M. Chatelain[1*], J. Gasparini[1] and A. Frantz[1]

[1] Sorbonne Universités, UPMC Univ Paris 06, UPEC, Paris 7, CNRS, INRA, IRD, Institut d'Ecologie et des Sciences de l'Environnement de Paris, F-75005, Paris, France

*Corresponding author: marion.chatelain@upmc.fr

Short title: metals, plumage colouration and immunity

ABSTRACT

Understanding the effects of trace metals emitted by anthropogenic activities on wildlife is of great concern in urban ecology; yet, information on how they affect individuals, populations, communities and ecosystems remains scarce. In particular, trace metals may impact survival by altering the immune system response to parasites. Melanin is assumed to influence the effects of trace metals on immunity owing to its ability to bind metal ions and the multiple pleiotropic effects of the gene coding its synthesis. We thus hypothesized that trace metals exposure would interact with plumage colouration in shaping immune response. We experimentally investigated the interaction between exposure to naturally-ranged concentrations of zinc and/or lead and melanin-based plumage colouration on components of the immune system in feral pigeons (Columba livia). First, we found experimental support for both immunostimulatory effects of zinc and immunotoxicity of lead. Second, we found weak interactive effects between trace metals exposure and plumage colouration on immune parameters. Nonetheless, pheomelanic pigeons exhibited a stronger humoral immune response than did eumelanic ones. Moreover, cellular immune response decreased with the plumage eumelanin level of lead-exposed birds. Both results may explain the scarcity of pheomelanic pigeons and the high frequency of darker eumelanic ones observed in urban areas. Indeed, weaker costly immune response may be beneficial when the probability of being parasitized is high, such as in the cities. Overall, our study points out the effects of trace metals on birds' immunity independently from other confounding urbanization factors, and underlines the need to investigate their impacts on other life history traits and their consequences in the ecology and evolution of host-parasite interactions.

Key words: ecotoxicology, immunoecology, melanin plumage

INTRODUCTION

Trace metals are largely emitted by anthropogenic activities such as the combustion of fossil energies by traffic and heating systems or metallurgic industries (Azimi et al. 2005). Consequently, trace metal concentrations in the biosphere are much higher nowadays than in the past (Thevenon et al. 2011, Sun et al. 2012). Zinc, lead, copper, chrome and magnesium are among the most abundant metals in atmosphere and in soil (Azimi et al. 2005, Maas et al. 2010). Their biological effects are various, ranging from beneficial to detrimental, and often depend on their concentrations (Hsu et al. 2006). For example, an experimental exposure to low concentrations of zinc is associated with a higher immune response in broilers (Smith 2003), whereas high concentrations of zinc induce neurological disorders in humans (Greenberg and Briemberg 2004); lead negatively affects broiler chickens growth (Bakalli et al. 1995) and induces the formation of reactive oxygen species in birds (reviewed in Koivula and Eeva 2010). Because of their physiological impact, either positive or negative, trace metals may affect life history traits of exposed organisms. However, most of the previous experimental studies scrutinized the effects of a single metal, while in the wild, individuals are exposed to a cocktail of metals. Moreover, previous experimental studies all investigated the effect of acute exposure to trace metals (Grasman and Scanlon 1995, Youssef et al. 1996, Vodela et al. 1997, Dauwe, L. Bervoets, R. Blust, M. Ee 2002, Snoeijs et al. 2005), while individuals rather suffer chronic but low exposures in their natural habitat. Consequently, experimental studies are needed to precisely know the effects of naturally-ranged concentrations of trace metals on animal wildlife.

In particular, trace metals may affect the immune system. For instance, great tits living far from a metallurgic smelter exhibit a higher humoral immune response than individuals living next to the factory (Snoeijs et al. 2004). In humans, zinc administration increases the size of the thymus and the number of T lymphocytes (Chandra and Dayton 1982), while lead exposure induces the production of autoantibodies and decreases T-cell activity (Mishra 2009). In addition, trace metals may also indirectly shape immunity by affecting the release of stress-induced hormones such as glucocorticoids (Vyskočil et al. 1990, Wayland 2002, Cory-Slechta et al. 2004, Virgolini 2005, Schulz et al. 2006, Baos et al. 2006, Franceschini et al. 2009, Herring et al. 2012), known to have immunosuppressive effects (Saino et al. 2003, Rubolini et al. 2005). Both direct and indirect effects of trace metals on immunity may affect survival by modulating the ability to fight against endo- and ecto-parasites. We previously found a positive and a negative relationship between endoparasites intensity and concentrations of zinc and lead in the feathers respectively,

while no link was observed with copper and cadmium (Gasparini et al. 2014). This observation suggests a particular importance of lead and zinc in shaping birds' immunity.

Melanin is the most widespread pigment in the animal reign. It exists in two types: eumelanin and pheomelanin, responsible for the black and reddish colours of teguments respectively. Variation in melanin-based plumage colouration is mainly genetically determined (Roulin 2004) and is correlated with diverse biological traits (including immunity-linked characteristics and stress response) due to a pleiotropic gene (POMC gene; Ducrest et al. 2008) or linkage disequilibrium (Mckinnon and Pierotti 2010). Previous work on feral pigeons showed that darker melanic individuals exhibit a higher cellular immune response than do paler ones (Jacquin et al. 2011). In addition, studies demonstrated a negative relation between barn owl plumage eumelanin level and stress response (Almasi et al. 2010, 2012). Therefore, melanin-based plumage colouration may shape birds' tolerance to trace metals by reducing the stress they induce. Besides this genetic link between melanin and immunity, melanin-based plumage colouration may shape trace metals effects on animals by controlling metals circulating levels. Indeed, negatively-charged free carboxyl, hydroxyl and amine functions of melanin (eumelanin and pheomelanin) are known to bind positively charged particles such as trace metals *in vitro* (Larsson and Tjälve 1978, Bridelli and Crippa 2007). Accordingly, it has been recently shown that zinc concentrations in feathers increase with feather darkness in feral pigeons (Chatelain et al. 2014). Trace metals are assumed to be transferred from the bloodstream into the pigmented cells of feathers during feather growth (Burger 1993). Owing to its chelator effect, melanin may favour trace metal detoxification in birds by decreasing the concentrations of potentially noxious metals in the body. In this context, the interaction between melanin-based plumage colouration and trace metal exposure may influence animals' immunity.

To test this hypothesis, we carried out an experimental study where feral pigeons (*Columba livia*) were chronically exposed to concentrations of zinc and/or lead representative of the natural range of environmental concentrations measured in urban areas. We measured different parameters of resistance against pathogens including humoral and cellular acquired immunities, hemolysis and hemagglutination abilities, and endoparasite prevalence and intensity. We predicted negative and positive effects of lead and zinc on these immune parameters, respectively. Feral pigeon is an urban species displaying a high variation in melanin-based plumage colouration differing in both melanin type (pheo- and eumelanin) and level (Haase et al. 1992, Johnston and Janiga 1995). Indeed, plumage colouration, constant through life, can vary from total absence of pigmentation (white plumage) to full dark melanic colouration (black or red plumage). Moreover, feral pigeons moult during a particularly extended period of more

than six months (Johnston and Janiga 1995), which may enhance metal detoxification through the chelator effect of melanin. Therefore, we also examined the effects of metal exposures on the previously described immune parameters according to the type of melanin (pheo- vs eumelanin) and to the eumelanin level of the plumage. We expected birds' melanin-based plumage colouration to moderate the respective effects of zinc and lead on immunity.

METHODS

Subjects and housing

Free-living feral pigeons (*Columba livia*) were caught in February and March 2013 in several pigeons flocks within the Parisian agglomeration. A sample of 96 pigeons were chosen in such a way as to best equilibrate sex-ratio (48 males and 48 females genetically sexed) and melanin-based plumage coloration degree (see "Measurement of plumage colouration"). Pigeons were kept in 8 outdoor aviaries (3.10 m x 2.00 m x 2.40 m) at the CEREEP field station (Centre d'Ecologie Expérimentale et Prédictive-Ecotron Ile-de-France, UMS 3194, Ecole Normale Supérieure, Saint-Pierre-lès-Nemours, France). They were evenly distributed among aviaries according to their flock, gender and eumelanic plumage colouration intensity in such a way there was no confounding effects between aviaries and these variables (i.e. no statistically significant link between aviary and flock: $Chi^2=71.09$, df=70, P=0.441; gender: 6 males and 6 females per aviary; and eumelanic plumage colouration intensity : $F_{1,80}=0.38$, P=0.537) . Pheomelanic individuals were spared in only 6 aviaries because of their lower number (n=14). All pigeons were fed *ad libitum* with a mix of maize, wheat and peas. The aviaries were enriched with a bowl of water used for bathing and with branches as perches. Birds were individually identified with a numbered plastic ring. At the end of the experiment (i.e. after 9 months of captivity), birds were released back to the wild at their site of capture.

Measurement of plumage colouration

Different deposition rates of pheomelanin (red pigmentation) and eumelanin (black pigmentation) allow a great variability of feather colouration ranging from totally white to completely red/dark plumage. Although both melanin types (pheomelanin and eumelanin) can be expressed within an individual, the birds used in this study where visually either pheomelanic (red/brown) or eumelanic (black) and were categorized as such. Because the frequencies of unmelanized (white) and pheomelanic pigeons in Parisian populations are smaller than the frequency of eumelanic ones, we captured 14 pheomelanic birds and no unmelanized ones. Plumage eumelanin level (i.e. darkness level) was calculated for eumelanic birds by evaluating

the dark surface on their wings (number of black pixels/number of white pixels x 100) using Gimp software. Dark surface measurements were highly repeatable between photographs of the same individual (Jacquin et al. 2011) and plumage colouration analysis is assumed to be a good approximation of feathers melanin content (Gasparini et al. 2009). Plumage eumelanin level was ranging from 4.2 to 95.9%. Because we had few pheomelanic individuals we did not measure a reddishness level.

General procedure

Before the start of the experiment, birds were acclimatized during a period ranging from 2 to 7 weeks, depending on their capture date, to reduce potential individuals' differences due to birds' previous exposure to metals and to remove potential stress effects of capture. There was no relation between acclimatization length period and aviary number (Chi^2=65.27, df=63, P=0.398).

Each aviary was randomly assigned to one of the 4 treatments, resulting in 2 aviaries with 12 pigeons each (24 pigeons in total) per treatment. Treatments consisted of tap water supplemented with either lead (Lead acetate, 1ppm), zinc (Zinc sulphate, 10ppm), or zinc and lead (Lead acetate 1ppm and Zinc sulphate 10ppm), plus a control treatment (pure tap water). We chose these concentrations based on both lead blood concentrations measured in urban birds (ranging from 0,053 to 0,264ppm; Roux and Marra 2007) and the gastrointestinal absorption rate of lead in zebra finches (<10%) calculated from (Dauwe, L. Bervoets, R. Blust, M. Ee 2002). Water, supplemented or not with zinc and/or lead, was supplied *ad libitum* and replaced every other day both in drinking troughs and baths. Note that no pheomelanic pigeon was exposed to both lead and zinc. The efficiency of our supplementation protocol was tested by measuring lead and zinc concentrations in birds' blood and new feathers grown after synchronized plucking, 10 and 12 weeks after the start of the experiment respectively. Both blood and feathers were digested (Chatelain et al. 2014) and lead and zinc concentrations were measured by mass spectrometry (ICP-MS) and by optic emission spectrometry (ICP-OES), respectively. Lead and zinc blood concentrations were higher among lead-exposed ($F_{1,45}$=4,47, P=0.040) and zinc-exposed ($F_{1,67}$=5.52, P=0.022) birds respectively as compared to controls. In the feathers, while lead concentrations were significantly higher among lead-exposed birds than in controls ($F_{1,76}$=19.61, P<0.001), zinc concentrations among zinc-exposed birds were not significantly higher than among controls ($F_{1,76}$=2.13, P=0.149). Altogether, these results validated our protocol.

Assessment of birds' immune response

Humoral immune response

To test birds' ability to produce specific antibodies under trace metals' exposure depending on their plumage colouration, we measured birds' humoral immune response. After 6 weeks of treatment, all pigeons were subcutaneously-injected with 50µg of a Keyhole Limpet Hemocyanin solution (Hemocyanin from *Megathura crenulata*, Sigma Aldrich), which is an antigen that individuals never encountered in the wild. A second injection of KLH was done 4 weeks after the first one. A blood sample was taken just before the first injection to estimate birds' initial anti-KLH antibody concentrations, then every week during 8 weeks; 50 µl of blood were collected from the brachial vein and were immediately centrifuged. Plasma were separated and frozen until analysis. Anti-KLH antibody concentrations in plasma were measured using a sandwich enzyme-linked immunosorbent assay (ELISA), following the method described by Jacquin et al (2013a). Anti-KLH antibody levels in the plasma taken 1, 2, 3 and 4 weeks after the first injection represented the primary immune response, whereas anti-KLH antibody levels in the plasma taken 1, 2, 3 and 4 weeks after the second injection (i.e. 5, 6, 7 and 8 weeks after the first injection) represented the secondary immune response.

Cellular immune response

Seventeen weeks after the start of the experiment, we tested whether birds' immunocompetence differed depending on metal exposure and on plumage colouration through a measure of birds' cellular response to phytohaemagglutinin (PHA), according to the method described by Jacquin et al (2011), adapted from Smits et al (1999). The right wing-web was injected with 0.1 ml of a 5 mg/ml PHA-P solution (Lectin from *Phaseolus vulgaris*, Sigma Aldrich) diluted in phosphate-buffered saline. Cellular response was calculated as wing-web thickness (mean of three successive measures) 24h after injection minus initial wing-web thickness (i.e. prior to injection) using a pressure-sensitive specimeter (Mitutoya 0-1mm). Due to logistical issues, birds from one treatment (exposed to both zinc and lead) were not injected with the PHA solution.

Natural antibodies and complement

Eleven weeks after the start of the experiment, plasma samples were also used to assess the ability to agglomerate and lyse rabbit blood cells owing to the hemolysis-hemagglutination assay using a protocol adapted from Matson et al (2005). Natural antibodies (agglutination ability) and complement (lyse ability) are both involved in the first line of protection against

invading microbes. Briefly, 25µl of plasma were distributed in the first and second well of each plate's line. Twenty-five µl of phosphate-buffered saline (PBS) were added to the second and following wells. Then, we did a warp dilution by transferring 25µl of the second well in the third one and so on (dilution from 1/2 to 1/256). Twenty-five µl of rabbit red blood cells (1%) were put in each well. Plates were softly shaken and incubating for 90 minutes at 37°C, then for 20 minutes at room temperature. Plates were visually read and scanned. Red blood cells were considered as non-agglutinated when we observed a red point in the bottom of the well, and as agglutinated when red blood cells were diffused. On the other hand, red blood cells were lysed when the wells appeared ochre and shiny. We obtained hemagglutination and hemolysis scores according to the maximum dilution at which we observed a positive well (e.g. a score of 3 signifies that we observed a positive well until dilution 1/4).

Haemosporidian parasites' prevalence and intensity

Haemosporidian (*Haemoproteus spp., Plasmodium spp.*) parasites' prevalence and intensity were measured from blood smears done both at the start of the experiment (t0) and after 4 weeks of treatment (t4). Slides were fixed with methanol during 5 minutes and coloured with GIEMSA (diluted 1:20) during 45 minutes. Individuals were considered as infected when at least one infected cell was detected. Parasite intensity was calculated among infected individuals as the number of infested red blood cells per 10000 red blood cells. Parasites identification was based on Hawkey and Denett (1989)

Statistical analyses

For each immune parameter, we first tested for the effect of metals exposures (lead exposure, zinc exposure and their interactions) on all the (pheo- and eumelanic) birds. Then, we tested for the effect of melanin type alone (pheo- and eumelanin) on these immune parameters. Finally, we focused on the eumelanic birds to test for the effects of eumelanin level in interaction with the exposure to metals on immune parameters; we did not perform such a model for pheomelanic birds because of their low sample size in the different treatments.

Humoral immune response was investigated using a linear mixed model with the (log-transformed) level of anti-KLH antibody as the dependent variable. We added the time of blood collection as covariate and aviary number and individual identity as random factors. Four individuals died during the experiment and were removed from this analysis. Cellular immune response (response to PHA) was tested using a linear mixed model with the aviary number as a

random factor. To investigate parasite prevalence (presence/absence of parasite) and (log-transformed) intensity, we performed generalized linear mixed models for binomial and Gaussian distribution respectively, with the time of blood collection as covariate and aviary number and individual identity as random factors. Hemagglutination and hemolysis scores were analysed using a general linear model for Poisson distribution.

Statistical analyses were performed using R software (version 3.0.2). Full models included all factors and covariates and all their interactions. We retained final models based on their AIC.

RESULTS

Effects of metal exposure

Metal exposure was not retained in the final models for the hemagglutination and lyse scores, nor for parasite prevalence and intensity.

When considering the humoral immune response (anti-KLH antibody level), we did find a significant interaction between time of blood collection, exposure to zinc and exposure to lead ($F_{8,91}$=2.0416.29, P=0.040; Fig. 1). At week 5, anti-KLH antibody levels were lower in lead-exposed pigeons compared to non lead-exposed birds ($F_{1,89}$=3.73, P=0.033). In contrast, at week 3 and 4 anti-KLH antibody levels were higher in zinc-exposed pigeons than in non zinc-exposed ones ($F_{1,88}$=1.91, P=0.063 and $F_{1,89}$=2.61, P=0.026 respectively). Besides, there was a significant interaction between zinc and lead exposure at weeks 6 ($F_{3,87}$=3.88, P=0.031) and 7 ($F_{3,86}$=3.25, P=0.042): the anti-KLH antibody levels were higher among individuals exposed to both zinc and lead as compared to controls (at week 6) and to individuals exposed to lead only (at week 6 and 7; Fig. 1).

Finally, zinc-supplemented pigeons tended to exhibit a higher cellular immune response (response to PHA) than non zinc-exposed birds ($F_{1,52}$=3.88, P=0.054).

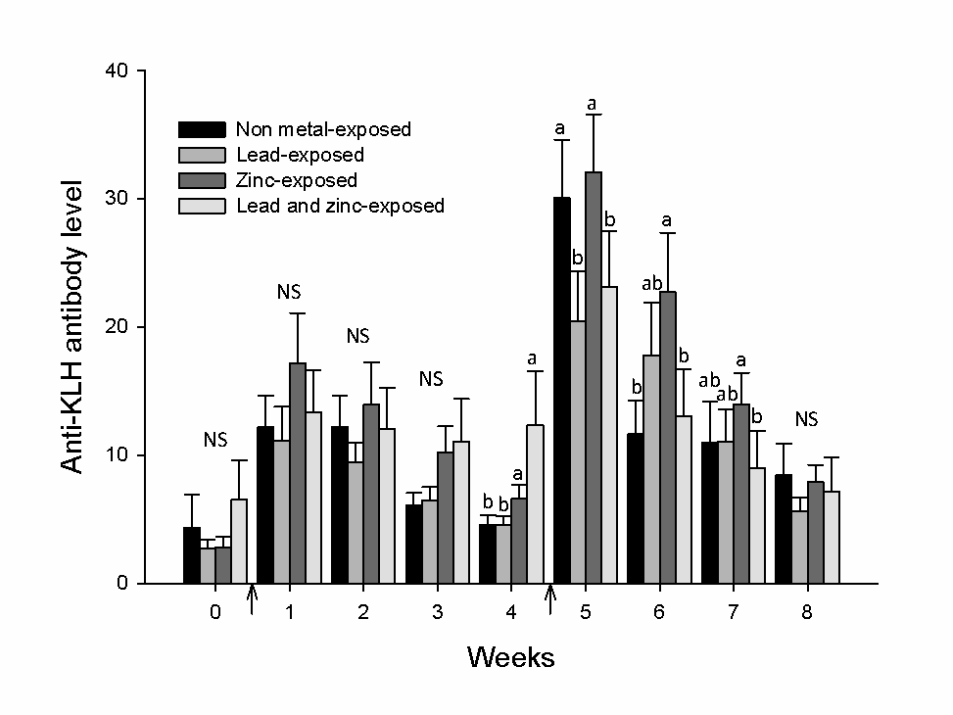


Fig. 1 Anti-KLH antibody levels (means ± se) in plasma among lead and/or zinc exposed birds over the weeks. Arrows represent the first and second KLH injections. Within a week, significant differences among metal exposure are indicated by different letters (a, b).

Effects of melanin type (pheomelanin vs. eumelanin)

Melanin type was not retained in the final models for hemagglutination score, lysis score, cellular immune response (response to PHA) and parasite prevalence and intensity.

However, there was a significant interaction between melanin type and the time of blood collection on the humoral immune response (anti-KLH antibody level; $F_{3,91}=2.17$, df=3, P=0.028; Fig. 2). More precisely, pheomelanic pigeons had a higher level of anti-KLH antibody than eumelanic ones at week 6 ($F_{1,90}=6.00$, P=0.014) and week 7 ($F_{1,89}=4.39$, P=0.036) while there was no such difference at any other week (all P-values > 0.125).

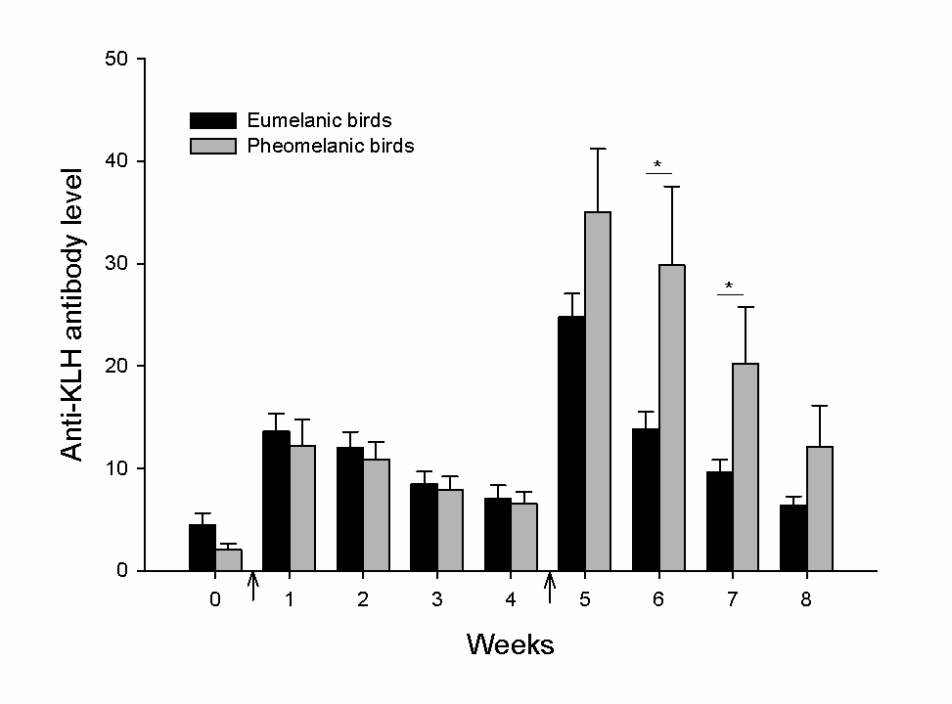


Fig. 2 Anti-KLH antibody levels (means ± se) in plasma of eumelanic (black bars) and pheomelanic birds (grey bars) over the weeks. Arrows represent the first and second KLH injections.

Effects of eumelanin level in interaction with metal exposure

Birds' eumelanin level (alone or in interaction with metal exposure) was not retained in the final models for the hemagglutination score, lysis scores, parasite prevalence and intensity and humoral immune response (anti-KLH antibody level).

However, there was a significant interaction between lead exposure and birds' eumelanin level on the cellular immune response (response to PHA; $F_{4,37}=4.52$, $P=0.040$; Fig. 3): when lead-exposed, darker pigeons exhibited a reduced inflammatory response ($F_{1,17}=5.30$, $P=0.034$), while this relation was not observed among non lead-exposed birds ($F_{2,20}=0.65$, $P=0.430$). Note that the initial parasite prevalence, which was more elevated for more melanic birds, did not affect the significance of this interaction ($P=0.003$).

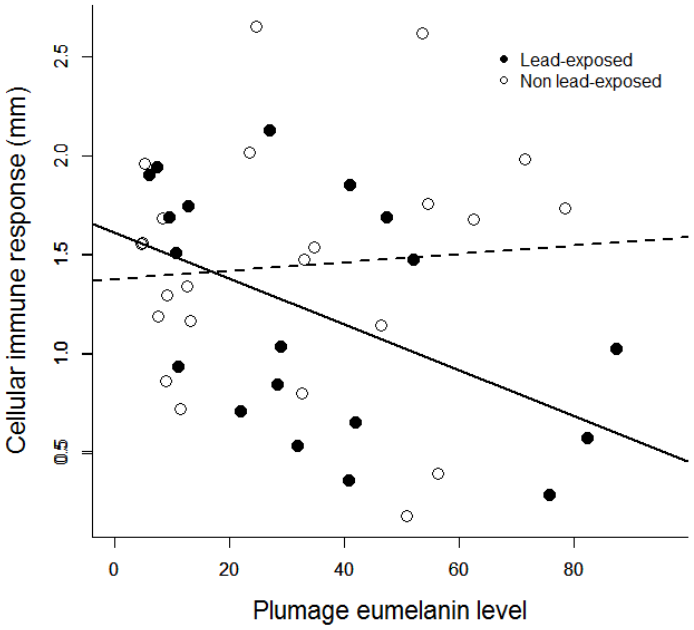


Fig. 3 Relationships between cellular immune response (difference of wing-web thickness prior and after 24h of PHA injection, in mm) and plumage eumelanin level (% of dark on their wing plumage) of lead-exposed (black dots and continuous regression line) and non lead-exposed birds (white dots and dashed regression line).

DISCUSSION

From an ecotoxicological point of view, our results show that naturally ranged concentrations of trace metals shape birds' immunity. While zinc increased birds' primary humoral and cellular immune responses, lead decreased the secondary humoral immune response. Accordingly, zinc is known to be essential in all aspects of immunity (Prasad 1998) and to be toxic for microorganisms (Babich and Stotzky 1978, Wirth et al. 1989), whereas lead has immunotoxic effects (Redig et al. 1991, Youssef et al. 1996, Snoeijs et al. 2005). However, our results show that the various aspects of the immune system (humoral and cellular immune response, parasite prevalence and intensity, natural antibodies and complement) do not systematically covary in response to an environmental factor and underline the need to conduct and generalise integrative studies in the future. Interestingly, we also found that lead-exposure masked the beneficial effect of zinc; indeed, the level of anti-KLH antibody decreased earlier in birds exposed to both zinc and lead as compared to birds exposed to zinc only. Our results thus provide experimental support for a recent study that found parasite intensity to be negatively

correlated to zinc exposure, but positively correlated to lead exposure in pigeons (Gasparini et al. 2014).

Further studies are needed to better understand the biological significance of trace metals effects on parasite resistance. In natura, birds are exposed to various concentrations of lead and zinc, as levels of these trace metals vary across space and time (Azimi et al. 2005, Scheifler et al. 2006, Roux and Marra 2007, Frantz et al. 2012). This spatio-temporal environmental heterogeneity is likely to affect populations' dynamics (i.e. population size and habitat use), as well as to select for different behaviours or physiological mechanisms for both hosts and parasites. Altogether, these findings underline the importance of trace metals in the ecology and evolution of host-parasite interactions.

Unexpectedly, our results demonstrated weak interactive effects between trace metals exposure and plumage colouration on immune parameters. More particularly, darker eumelanic pigeons did not exhibit a higher cellular immune response than did paler birds, even among controls, though such a relationship has been previously observed in this species (Jacquin et al. 2011). This may be due to food supply, which was ad-libitum in our study. Our experimental design could have shaded the variation in birds' ability to cope with food availability, shown to depend on birds' melanin-based plumage colouration (Jacquin et al. 2012) and known to affect immunity (Lochmiller and Deerenberg 2000). Nevertheless, we observed an interaction between birds' eumelanin level and lead-exposure on cellular immune response; when lead-exposed, darker eumelanic birds displayed a lower cellular immune response than did paler ones. Interestingly, the direction of this relation is opposite to our prediction of a beneficial role of melanin under lead exposure: plumage eumelanin level was associated with increased negative effect of lead on the immune response. The underlying mechanisms remain to be elucidated but might involve some trade-off between eumelanogenesis and other biological functions (detoxification, stress response, etc.). Moreover, it is unclear whether a weak or a high immune response would be selected according to the probability of being parasitized. Although parasite resistance is positively linked to survival (Møller and Saino 2004, Cichon and Dubiec 2005), a low immune response may allow to spare energy allocable to other life history traits (Bonneaud et al. 2003). In cities, infection prevalence and intensity in birds are the highest (Jacquin et al. 2013b, Giraudeau et al. 2014). These habitats are also characterized by significant pollution levels, including trace metals (Azimi et al. 2005, Roux and Marra 2007), costly for animals to cope with. The high probability to be infected in cities may lead to a costly over-stimulation of the immune system (Lochmiller and Deerenberg 2000, Hanssen et al. 2004, Eraud et al. 2009). Therefore, from an evolutionary point of view, the lower cellular immune response of darker

eumelanic pigeons' to lead-exposure may be adaptive and allow them to spare energy to support the costs of trace metals detoxification. Interestingly, we also found an association between melanin-type (pheomelanin vs. eumelanin) and humoral immunity; pheomelanic pigeons displayed a higher secondary immune response than did eumelanic ones. Similarly, the reddishness of females' tawny owls positively correlates with their humoral immune response (Gasparini et al. 2009). Although we did observe an effect of melanin type on only one of the immune parameters measured, our results suggest that pheomelanic birds may allocate more energy to cope with pathogens and parasites than do eumelanic birds. Both the lower cellular immune response of darker eumelanic pigeons and the higher humoral immune response of pheomelanic birds compared to eumelanic ones may partly explain why eumelanic pigeons are more common than pheomelanic ones in the cities (Obukhova 2007) respectively (but see Galván and Møller 2013).

While trace metals consistently affect immune response and may consequently represent a new selective pressure favouring more tolerant phenotypes, highly melanic plumage seems not to be such an advantageous phenotype, Indeed, only one interaction was measured between plumage eumelanin level and lead exposure on immunity, suggesting that trace metals would be only slightly involved in melanin-based plumage colouration evolution. However, the benefits of intense melanic plumage colouration to cope with trace metals have to be investigated on other biological parameters, such as survival and reproduction success. Moreover, we have to investigate such potential effect on a longer period; indeed, although feral pigeons moult all over the year, metals detoxification, one of the beneficial effect of plumage melanism hypothesized in this study, may occur during juveniles' growth and during fall (i.e. when moulting is at a peak). Finally, we found weak differences between eumelanic and pheomelanic pigeons immunity, which suggest that variability of immune response would not significantly explain plumage colouration frequencies.

ACKNOWLEDGMENTS

All experiments were conducted under the authorizations of the "Ministère de l'éducation nationale, de l'enseignement supérieur et de la recherche" (authorization N_00093.02) and the "Direction Départementale des Services Vétérinaires de Seine et-Marne" (authorization N_ 77-05). We thank the "Mairie de Paris" (Thomas Charachon) for allowing the capture of birds. The Centre de Recherche en Ecologie Expérimentale et Prédictive (CEREEP) provided logistic support

for the field work of this study. We thank S. Leclaire, T. Gayet, S. Pollet, S. Hasnaoui, F. Lorente, S. Perret and B. Decencière for their help all along the field work and C. Haussy for the realisation of the laboratory work. We testify that this study does not raise any conflict of interest.

REFERENCES

Almasi, B. et al. 2010. Regulation of stress response is heritable and functionally linked to melanin-based coloration. - J. Evol. Biol. 23: 987–996.

Almasi, B. et al. 2012. Coloration signals the ability to cope with elevated stress hormones: effects of corticosterone on growth of barn owls are associated with melanism: Plumage coloration and stress sensitivity. - J. Evol. Biol. 25: 1189–1199.

Azimi, S. et al. 2005. Sources, distribution and variability of hydrocarbons and metals in atmospheric deposition in an urban area (Paris, France). - Sci. Total Environ. 337: 223–239.

Babich, H. and Stotzky, G. 1978. Toxicity of zinc to fungi, bacteria, and coliphages: influence of chloride ions. - Appl. Environ. Microbiol. 36: 906–914.

Bakalli, R. I. et al. 1995. The magnitude of lead toxicity in broiler chickens. - Vet. Hum. Toxicol. 37: 15–19.

Baos, R. et al. 2006. Adrenocortical response to stress and thyroid hormone status in free-living nestling white storks (*Ciconia ciconia*) exposed to heavy metal and arsenic contamination. - Environ. Health Perspect. 114: 1497–1501.

Bonneaud, C. et al. 2003. Assessing the cost of mounting an immune response. - Am. Nat. 161: 367–379.

Bridelli, M. G. and Crippa, P. R. 2007. Theoretical analysis of the adsorption of metal ions to the surface of melanin particles. - Adsorption 14: 101–109.

Burger, J. 1993. Metals in avian feathers: bioindicators of environmental pollution. - Rev. Environ. Contam. Toxicol. 5: 203–311.

Chandra, R. K. and Dayton, D. H. 1982. Trace element regulation of immunity and infection. - Nutr. Res. 2: 721–733.

Chatelain, M. et al. 2014. The adaptive function of melanin-based plumage coloration to trace metals. - Biol. Lett. 10: 20140164–20140164.

Cichon, M. and Dubiec, A. 2005. Cell-mediated immunity predicts the probability of local recruitment in nestling blue tits. - J. Evol. Biol. 18: 962–966.

Cory-Slechta, D. A. et al. 2004. Maternal stress modulates the effects of developmental lead exposure. - Environ. Health Perspect. 112: 717–730.

Dauwe, L. Bervoets, R. Blust, M. Ee, T. 2002. Tissue levels of lead in experimentally exposed zebra finches (*Taeniopygia guttata*) with particular attention on the use of feathers as biomonitors. - Arch. Environ. Contam. Toxicol. 42: 88–92.

Ducrest, A. et al. 2008. Pleiotropy in the melanocortin system, coloration and behavioural syndromes. - Trends Ecol. Evol. 23: 502–510.

Eraud, C. et al. 2009. Survival cost of an early immune soliciting in nature. - Evolution 63: 1036–1043.

Franceschini, M. D. et al. 2009. The corticosterone stress response and mercury contamination in free-living tree swallows, *Tachycineta bicolor*. - Ecotoxicology 18: 514–521.

Frantz, A. et al. 2012. Contrasting levels of heavy metals in the feathers of urban pigeons from close habitats suggest limited movements at a restricted scale. - Environ. Pollut. 168: 23–28.

Galván, I. and Møller, A. P. 2013. Pheomelanin-based plumage coloration predicts survival rates in birds. - Physiol. Biochem. Zool. 86: 184–192.

Gasparini, J. et al. 2009. Strength and cost of an induced immune response are associated with a heritable melanin-based colour trait in female tawny owls. - J. Anim. Ecol. 78: 608–616.

Gasparini, J. et al. 2014. Relationships between metals exposure and epidemiological parameters of two pathogens in urban pigeons. - Bull. Environ. Contam. Toxicol. 92: 208–212.

Giraudeau, M. et al. 2014. Parasites in the city: degree of urbanization predicts poxvirus and coccidian infections in house finches (*Haemorhous mexicanus*) (JD Brown, Ed.). - PLoS ONE 9: e86747.

Grasman, K. A. and Scanlon, P. F. 1995. Effects of acute lead ingestion and diet on antibody and T-cell-mediated immunity in japanese quail. - Arch. Environ. Contam. Toxicol. 28: 161–167.

Greenberg, S. A. and Briemberg, H. R. 2004. A neurological and hematological syndrome associated with zinc excess and copper deficiency. - J. Neurol. 251: 111–114.

Haase, E. et al. 1992. Melanin concentrations in feathers from wild and domestic pigeons. - J. Hered. 83: 64–67.

Hanssen, S. A. et al. 2004. Costs of immunity: immune responsiveness reduces survival in a vertebrate. - Proc. R. Soc. B Biol. Sci. 271: 925–930.

Hawkey, C. M. and Denett, T. B. 1989. A colour atlas of comparative veterinary haematology.

Herring, G. et al. 2012. Mercury exposure may suppress baseline corticosterone levels in juvenile birds. - Environ. Sci. Technol. 46: 6339–6346.

Hsu, M. J. et al. 2006. Taiwan's industrial heavy metal pollution threatens terrestrial biota. - Environ. Pollut. 143: 327–334.

Jacquin, L. et al. 2011. Melanin-based coloration is related to parasite intensity and cellular immune response in an urban free living bird: the feral pigeon *Columba livia*. - J. Avian Biol. 42: 11–15.

Jacquin, L. et al. 2012. Melanin-based coloration reflects alternative strategies to cope with food limitation in pigeons. - Behav. Ecol. 23: 907–915.

Jacquin, L. et al. 2013a. Darker female pigeons transmit more specific antibodies to their eggs than do paler ones. - Biol. J. Linn. Soc. 108: 647–657.

Jacquin, L. et al. 2013b. A potential role for parasites in the maintenance of color polymorphism in urban birds. - Oecologia 173: 1089–1099.

Johnston, R. and Janiga, M. 1995. Feral pigeons. - Oxford University Press.

Koivula, M. J. and Eeva, T. 2010. Metal-related oxidative stress in birds. - Environ. Pollut. 158: 2359–2370.

Larsson, B. and Tjälve, H. 1978. Studies on the melanin-affinity of metal ions. - Acta Physiol. Scand. 104: 479–484.

Lochmiller, R. L. and Deerenberg, C. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? - Oikos 88: 87–98.

Maas, S. et al. 2010. Spatial distribution of heavy metal concentrations in urban, suburban and agricultural soils in a Mediterranean city of Algeria. - Environ. Pollut. 158: 2294–2301.

Matson, K. D. et al. 2005. A hemolysis–hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. - Dev. Comp. Immunol. 29: 275–286.

Mckinnon, J. S. and Pierotti, M. E. R. 2010. Colour polymorphism and correlated characters: genetic mechanisms and evolution. - Mol. Ecol. 19: 5101–5125.

Mishra, K. P. 2009. Lead exposure and its impact on immune system: A review. - Toxicol. In Vitro 23: 969–972.

Møller, A. P. and Saino, N. 2004. Immune response and survival. - Oikos 104: 299–304.

Obukhova, N. 2007. Polymorphism and phene geography of the blue rock pigeon in Europe. - Russ J Genet 43: 492–501.

Redig, P. T. et al. 1991. Effects of chronic exposure to sublethal concentrations of lead acetate on heme synthesis and immune function in red-tailed hawks. - Arch. Environ. Contam. Toxicol. 21: 72–77.

Roulin, A. 2004. The evolution, maintenance and adaptive function of genetic colour polymorphism in birds. - Biol. Rev. 79: 815–848.

Roux, K. E. and Marra, P. P. 2007. The presence and impact of environmental lead in passerine birds along an urban to rural land use gradient. - Arch. Environ. Contam. Toxicol. 53: 261–268.

Rubolini, D. et al. 2005. Effects of elevated egg corticosterone levels on behavior, growth, and immunity of yellow-legged gull (*Larus michahellis*) chicks. - Horm. Behav. 47: 592–605.

Saino, N. et al. 2003. Immune response covaries with corticosterone plasma levels under experimentally stressful conditions in nestling barn swallows (*Hirundo rustica*). - Behav. Ecol. 14: 318–325.

Scheifler, R. et al. 2006. Lead concentrations in feathers and blood of common blackbirds (*Turdus merula*) and in earthworms inhabiting unpolluted and moderately polluted urban areas. - Sci. Total Environ. 371: 197–205.

Schulz, J. H. et al. 2006. Acute lead toxicosis in mourning doves. - J. Wildl. Manag. 70: 413–421.

Smith, M. O. 2003. Effects of different levels of zinc on the performance and immunocompetence of broilers under heat stress. - Poult. Sci. 82: 1580–1588.

Smits, J. E. et al. 1999. Simplifying the phytohaemagglutinin skin-testing technique in studies of avian immunocompetence. - Funct. Ecol. 13: 567–572.

Snoeijs, T. et al. 2004. Heavy metal exposure affects the humoral immune response in a free-living small songbird, the great tit (*Parus major*). - Arch. Environ. Contam. Toxicol. in press.

Snoeijs, T. et al. 2005. The combined effect of lead exposure and high or low dietary calcium on health and immunocompetence in the zebra finch. - Environ. Pollut. 134: 123–132.

Sun, Q. et al. 2012. Temporal and spatial distribution of trace metals in sediments from the northern Yellow Sea coast, China: implications for regional anthropogenic processes. - Environ. Earth Sci. 66: 697–705.

Thevenon, F. et al. 2011. Local to regional scale industrial heavy metal pollution recorded in sediments of large freshwater lakes in central Europe (lakes Geneva and Lucerne) over the last centuries. - Sci. Total Environ. 412-413: 239–247.

Virgolini, M. B. 2005. Interactions of chronic lead exposure and intermittent stress: consequences for brain catecholamine systems and associated behaviors and HPA axis function. - Toxicol. Sci. 87: 469–482.

Vodela, J. K. et al. 1997. Drinking water contaminants (arsenic, cadmium, lead, benzene, and trichloroethylene). 1. Interaction of contaminants with nutritional status on general performance and immune function in broiler chickens. - Poult. Sci. 76: 1474–1492.

Vyskočil, A. et al. 1990. Influence of chronic lead exposure on hormone levels in developing rats. - J. Appl. Toxicol. 10: 301–302.

Wayland, M. 2002. Immune function, stress response, and body condition in arctic-breeding common eiders in relation to cadmium, mercury, and selenium concentrations. - Environ. Res. 90: 47–60.

Wirth, J. J. et al. 1989. Zinc requirement for macrophage function: effect of zinc deficiency on uptake and killing of a protozoan parasite. - Immunology 68: 114.

Youssef, S. A. . et al. 1996. Effect of subclinical lead toxicity on the immune response of chickens to Newcastle disease virus vaccine. - Res. Vet. Sci. 60: 13–16.

Chapitre 4 :

Métaux traces, mélanisme et transferts maternels précoces

Introduction

L'expérience conduite en 2013 nous a permis de mettre en évidence des effets négatifs et positifs du plomb et du zinc sur plusieurs paramètres de la reproduction. Notamment, les juvéniles exposés au plomb seul montraient une croissance écourtée et une plus petite corpulence à 3 mois. Ces effets peuvent résulter d'un effet direct des métaux sur les juvéniles du fait d'une exposition via l'alimentation (i.e. le lait de jabot dans les premiers jours, puis la nourriture et l'eau mise à disposition). Ils peuvent aussi être dus à des effets des métaux sur la condition des parents, qui modifieraient en conséquence les effets parentaux dont notamment les transferts maternels précoces. En effet, chez les ovipares, la mère transfère dans ses œufs des éléments divers comme des hormones (e.g. testostérone, corticostérone, prolactine, etc. ; Groothuis and Schwabl, 2008), des nutriments (Naber, 1979), des éléments participant à l'immunité des œufs et des juvéniles (Boulinier and Staszewski, 2008; Hasselquist and Nilsson, 2009) ou encore des xénobiotiques (Agusa et al., 2005). Les transferts de composants de l'immunité sont notamment essentiels à l'éclosion de l'œuf et à la survie et la croissance du juvénile (Heller et al., 1990; Pihlaja et al., 2006; Saino et al., 2002), celui-ci étant à l'éclosion immunonaïf (Dibner et al., 1998; Mauck et al., 2005), c'est-à-dire qu'il n'est pas capable de produire ses propres anticorps. Ces transferts peuvent être coûteux pour la mère (Hargitai et al., 2006) ; aussi, les métaux traces, en modifiant l'état physiologique de la mère, sont susceptibles d'affecter les transferts maternels précoces (i.e. dans les œufs). Cette hypothèse pourrait permettre d'expliquer les effets des métaux traces observés sur le succès d'éclosion, la survie et la croissance des juvéniles (Eeva et al., 2009; Eeva and Lehikoinen, 1996; Chatelain et al. soumis; Sens et al., 2003).

Par ailleurs, le mélanisme du plumage étant potentiellement lié à l'immunité via des effets pléiotropes (Ducrest et al., 2008), et à la capacité à détoxifier les métaux traces, la coloration mélanique du plumage est susceptible d'affecter les transferts maternels précoces de composants de l'immunité et de moduler les effets des métaux traces sur ces transferts. D'ailleurs, des travaux précédents sur le pigeon biset montrent un lien positif entre le degré d'eumélanisme du plumage et le transfert d'anticorps spécifiques (Jacquin et al., 2013).

Méthodes

Durant l'étude expérimentale réalisée en 2014 (étude expérimentale 2), l'ensemble des femelles ont reçu une injection de KLH (keyhole limpet hemocyanin), un antigène auquel les pigeons n'ont jamais été exposés dans leur milieu naturel. Le taux en anticorps anti-KLH a été mesuré dans les œufs (i.e. dans chaque ponte, le 1er ou le 2nd œuf pondu a été prélevé de façon aléatoire et congelé), ainsi que chez la mère au moment de la ponte. Les concentrations en lysozymes et en ovotransferrine ont également été mesurées dans l'albumen des œufs prélevés.

Conclusion

Dans un premier temps notre étude montre un effet négatif de l'exposition au plomb sur le transfert d'anticorps anti-KLH. Les transferts maternels déterminant la capacité les jeunes poussins à combattre les infections, l'inhibition du transfert d'anticorps par le plomb peut en partie expliquer la forte mortalité des poussins exposés aux métaux traces et l'altération de la croissance des survivants observée chez les mésanges bleues et charbonnières (Eeva et al., 2009; Eeva and Lehikoinen, 1996) et chez le pigeon biset (Chatelain et al. soumis). De plus, nos résultats suggèrent que l'exposition au plomb augmente le transfert de l'ovotransferrine, connue pour chélater certains ions métalliques dont quelques-uns sont nécessaires à la croissance de bactéries et champignons (Valenti et al., 1985, 1983). Aussi, de plus forts taux en ovotransferrine pourraient permettre une meilleure inhibition de la croissance microbienne. Néanmoins, le rôle de l'ovotransferrine est assez mal connu et ses concentrations pourraient également représenter l'état d'infection et d'inflammation de la mère (Horrocks et al., 2011), ou encore la capacité à détoxifier certains métaux, comme le plomb, par leur inactivation (Pohanka et al., 2012). L'effet positif d'une exposition au plomb sur son transfert semble plutôt aller dans le sens de cette dernière hypothèse. La plasticité dans le transfert de l'ovotransferrine pourrait ainsi permettre l'acclimatation à des concentrations environnementales élevées en métaux traces. Aussi, il serait particulièrement intéressant de tester de façon plus précise comment les concentrations en métaux chez la mère module le transfert de l'ovotransferrine, tout en contrôlant par son état physiologique.

Finalement, notre étude montre que les femelles au plumage le plus eumélanique transfèrent davantage de lysozymes, molécule catalysant la lyse des parois cellulaires des bactéries Gram+, par rapport aux femelles au plumage plus clair. Cependant, cet effet est masqué chez les individus exposés au zinc, suggérant un effet bénéfique du zinc sur les femelles les plus clairs uniquement. Les femelles les plus foncés transfèrent davantage de zinc dans leurs plumes

(Chatelain et al., 2014) et celui-ci serait alors moins disponible pour certains processus biologiques tels que l'immunité (Prasad, 1998). De ce fait, dans le cas où les concentrations environnementales en zinc, et peut-être plus généralement en métaux traces essentiels, seraient limitantes, les œufs issus de femelles au plumage davantage eumélanique disposeraient de concentrations en lysozymes plus élevées et par conséquent auraient une plus forte probabilité d'éclosion (Saino et al., 2002). Au contraire, dans le cas où le zinc serait abondant dans l'habitat, par exemple en milieu urbain (Azimi et al., 2003), les œufs issus des femelles les plus claires auraient de meilleures probabilités d'éclosion. Néanmoins, aucune différence de taux d'éclosion n'a été mise en évidence lors de l'étude expérimentale 1 ; aussi, l'avantage sélectif que pourrait représenter la coloration mélanique des femelles selon les concentrations environnementales en zinc reste à être évalué.

Ce chapitre prend la forme d'un article soumis dans Physiological and Biochemical Physiology.

Trace metals affect early maternal transfer of immune components in the feral pigeon

Chatelain M[1*], Gasparini J[1], Haussy C[1] and Frantz A[1]

[1] Sorbonne Universités, UPMC Univ Paris 06, UPEC, Paris 7, CNRS, INRA, IRD, Institut d'Ecologie et des Sciences de l'Environnement de Paris, F-75005, Paris, France

*Author for correspondence: marion.chatelain@upmc.fr

Running title: trace metals and maternal transfers

ABSTRACT

Maternal early transfers of immune components influence eggs' hatching probability and nestlings' survival. They depend on females' own immunity and, because costly, on their condition. Therefore, trace metals, whether toxic and immunosuppressive (e.g. lead, cadmium, etc.) or necessary and immunostimulant (e.g. zinc, copper, iron, etc.) are likely to affect the amount of immune components transferred into the eggs. It may also vary with plumage eumelanin level, known to be linked to immunity, to antibodies transfer and to metals detoxification. In feral pigeons (Columba livia) injected with an antigen and experimentally exposed to lead and/or zinc, two highly abundant trace metals in urban areas, we measured specific antibodies transfer and concentrations of two antimicrobial proteins (lysozyme and ovotransferrin) in eggs. As expected, lead had negative effects on specific antibodies transfer, while zinc positively affected lysozyme eggs concentrations. Moreover, eggs from lead-exposed females exhibited higher ovotransferrin concentrations; because it binds metal ions, ovotransferrin may enable eggs detoxification and embryo protection. Finally, eggs' lysozyme concentrations increased with plumage darkness of zinc non-exposed females, while the relation is opposite among zinc-exposed females, suggesting that benefits and costs of plumage melanism depend on trace metals environmental levels. Overall, our study underlines the potential ecotoxicological effects of trace metals on reproductive success and the role of plumage melanism in modulating these effects.

Key words: maternal effects, immunity, ecotoxicology, urban pollution, plumage colouration, eumelanin

INTRODUCTION

At birth, offspring are immunologically naive as they do not produce their own antibodies (Dibner et al., 1998; Mauck et al., 2005), and are consequently highly vulnerable to parasites. Specific antibodies transferred into egg yolk may increase offspring's ability to cope with parasites (Gasparini et al., 2006) and therefore to improve their survival early in life (Heller et al., 1990; Pihlaja et al., 2006). Furthermore, antimicrobial proteins, such as lysozyme and ovotransferrin, are also transferred into egg albumen and are consequently part of egg innate immune system. Lysozyme catalyses the lysis of gram-positive bacteria by hydrolysing the peptidoglycan in their cell walls (Masschalck and Michiels, 2003; Pellegrini et al., 1992), while ovotransferrin inhibits microbes' growth by chelating metallic ions necessary for their development (Fe^{3+}, Cu^{2+}, Zn^{2+}; Valenti et al., 1983; Valenti et al., 1985). It has been shown that the transfer of such proteins may enhance offspring survival by decreasing egg hatching failure (Saino et al., 2002a).

The amount of immune components in eggs depends both on female's production (Jacquin et al., 2013; Saino et al., 2002a) and on the proportion she allocates to her eggs. Indeed, it has been recently suggested that the investment of immune substances into the egg was costly to mothers (Ismail et al., 2015) and may depend on the maternal environment. For instance, maternal transfer of antibodies depends on female's condition (Hargitai et al., 2006). Therefore, any environmental factor that influences female's own immunity and/or entails costs or benefits for females should impact the transfer of immune substances into the eggs.

Trace metals (lead, zinc, cadmium, etc.) are particularly abundant in urban areas (Azimi et al., 2003; Roux and Marra, 2007) and may induce noxious effects on bird physiology (Dauwe et al., 2005; Eeva et al., 2009; Redig et al., 1991; Snoeijs et al., 2004). Therefore, trace metals exposure may entail significant costs for females that consequently reduce the investment of the immune components into the eggs (Hargitai et al., 2006; Saino et al., 2002b). Alternatively, from an adaptive point of view, mothers may adjust their investment into the egg in response to the stress induced by metal exposure to prepare the offspring to future environment (Gasparini et al., 2007).

To examine whether and how trace metals exposure may shape early maternal transfer of immune components, we chronically exposed feral pigeons (C*olumba livia*) to concentrations of zinc and/or lead representative of the natural environmental range measured in urban areas. Lead and zinc are among the most abundant metals in atmosphere and in soil of urban areas (Azimi et al., 2005; Maas et al., 2010). Lead is a well-known toxic metal (Jarup, 2003; Patrick,

2006) and an immunosuppressor (Gasparini et al., 2014; Redig et al., 1991; Snoeijs et al., 2004; Snoeijs et al., 2005; Trust et al., 1990), while zinc has beneficial effects on all aspects of immunity (Gasparini et al., 2014; Prasad, 1998; Prasad, 2009; Smith, 2003). Therefore, both lead and zinc may modulate immune components transfer by affecting both females' immunity and females' reproductive investment. We estimated early maternal transfer of immune components: specific antibodies, lysozymes and ovotransferrins. We expected lead and zinc exposure to respectively reduce and increase immune component transfer, associated to costs and benefits on females. Alternatively, opposite results may suggest a strategical paternal response.

Interestingly, antibodies transfer from females feral pigeons to their young increased with female plumage darkness (Jacquin et al., 2013), potentially due to genetic links between melanogenesis and immunity (Ducrest et al., 2008) or to the detoxification ability of melanin (Chatelain et al., 2014). Thus, plumage melanism may affect the transfer of immune components and modulate the impacts of trace metals on this transfer.

RESULTS

Two models were equally parsimonious (their AIC were not significantly different: 105.8 and 104.4 respectively). In the first one, anti-KLH antibodies level was lower in eggs laid by lead-exposed mothers (*lead* and *lead+zinc* groups) than in the others (*control* and *zinc* groups; Table 1; 7.38±1.61 and 11.78±2.62 respectively). In the second one, egg anti-KLH antibodies level increased with the mother anti-KLH antibodies level (Table 1; Fig. 1).

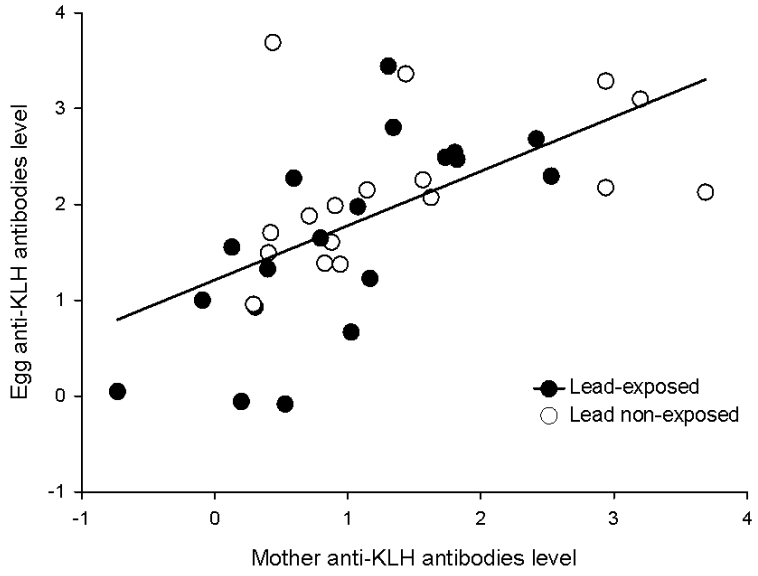


Fig. 1 Log-transformed egg anti-KLH antibodies level according to log-transformed mother anti-KLH antibodies level.

Lysozyme concentration depended on the interaction between zinc exposure and mother eumelanin level (Table 1; Fig. 2): lysozyme concentration was negatively linked with maternal eumelanin level in egg from zinc-exposed mothers (*zinc* and *lead+zinc* groups; $F_{1,18}$=5.70, P=0.017), while it was positively linked with maternal eumelanin level in egg from zinc non-exposed mothers (*control* and *lead* groups; $F_{1,22}$=4.42, P=0.035).

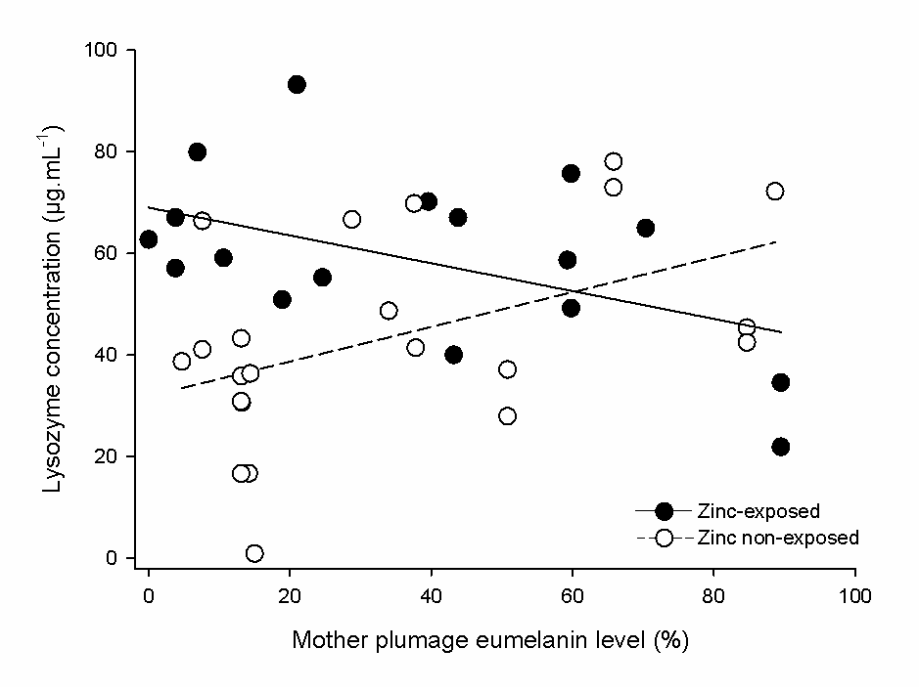


Fig. 2 Egg lysozyme concentration (µg.mL^{-1}) according to females' plumage eumelanin level (%).

Ovotransferrin concentration depended on the interaction between zinc exposure and lead exposure (Table 1; Fig. 3): eggs laid by mothers exposed to lead only (*lead* group) had higher ovotransferrin concentrations than eggs laid by mothers exposed to both lead and zinc (*lead+zinc* group; $F_{1,25}$=7.37, P=0.007).

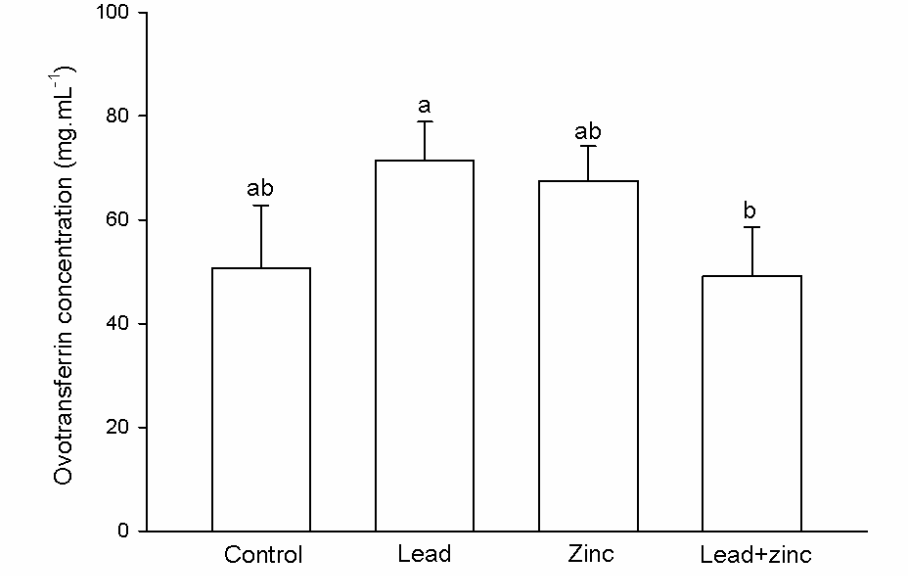


Fig. 3 Egg ovotransferrin concentration (mg.mL^{-1}) according to the exposure to lead and/or zinc.

	Anti-KLH antibodies	*Lysozymes*	*Ovotransferrins*
Zinc exposure	-	$F_{1,40}$=7.44; P=0.006 **	$F_{1,49}$=1.25; P=0.263
Lead exposure	$^{(1)}F_{1,38}$=3.93; P=0.046 *	$F_{1,40}$=0.06; P=0.814	$F_{1,49}$=2.41; P=0.121
Mother eumelanin level	-	$F_{1,40}$=11.45; P<0.001 ***	-
Mother antibodies level	$^{(1)}F_{1,38}$=2.67; P=0.102 $^{(2)}F_{1,38}$=14.21; P<0.001 ***		
Zinc exposure x Lead exposure	-	$F_{1,40}$=0.42; P=0.517	$F_{1,49}$=4.32; P=0.037 *
Zinc exposure x Mother eumelanin level	-	$F_{1,40}$=14.67; P<0.001 ***	-
Lead exposure x Mother antibodies level	$^{(1)}F_{1,38}$=1.90; P=0.169		

Tab. 1 *Final linear mixed-effects model ANOVAs with log-transformed egg anti-KLH antibodies level, lysozyme concentration or ovotransferrin concentration as the dependent variable, zinc exposure, lead exposure, mother eumelanin level, mother anti-KLH antibodies level when relevant and their interactions as the explanatory variables and the aviary and mother identity as random factors. For anti-KLH antibodies transfer,* two models were equally parsimonious *and are mentioned as (1) and (2).*

DISCUSSION

Our aim was first to investigate the effects of a chronic exposure to trace metals in concentrations encountered in urban areas on early maternal transfer (from mother to eggs) of components influencing egg immune components (specific antibodies, lysozymes, ovotransferrin and metals) and second to test whether mother melanin-based plumage colouration modulates such effects.

In accordance with our hypothesis, the transfer of specific antibodies (anti-KLH antibodies) was smaller in eggs from lead-exposed mothers (*lead* and *lead+zinc* groups) than in the other eggs. This result underlines the negative effects of lead on egg immunity and may explain the higher hatching failure observed in blue and great tits nests exposed to high concentrations of trace metals (Eeva et al., 2009; Sens et al., 2003). Moreover, nestlings are immunologically naive and fight off parasites with their innate immune system inherited from their mother (Hasselquist and Nilsson, 2009). Therefore, coping with parasites may be more costly for nestlings with low maternal antibodies and may consequently explain the lower nestlings' growth and the higher nestling mortality also observed in blue tits, great tits and feral pigeons exposed to trace metals (Eeva and Lehikoinen, 1996; Eeva et al., 2009). Moreover, this result is in accordance with our previous study demonstrating a negative effect of lead exposure on the second humoral immune response (anti-KLH antibodies level) in adult feral pigeons (Chatelain et al. unpublished).

In addition, ovotransferrin concentration was higher in eggs laid by mothers exposed to lead only (*lead* group) than in eggs laid by mothers exposed to both lead and zinc (*lead+zinc* group). First, this result suggests that lead exposure increases ovotransferrin transfer and second, that zinc exposure moderates the effect of lead exposure on ovotransferrin transfer due to its effects on lead absorption and lead binding (Cerklewski and Forbes, 1976; El-Gazzar et al., 1978; Godwin, 2001). Interestingly, ovotransferrin binds divalent ions including lead (Pb^{2+}; Pohanka et al., 2012). Higher ovotransferrin levels may therefore help detoxifying lead potentially transferred into the egg (Agusa et al., 2005) and may consequently reduce the noxious effects of lead on embryonic development. From an adaptive point of view, this result may suggest an adaptive strategy of maternal transfers of ovotransferrin. More studies are needed to understand whether ovotransferrin concentrations in eggs reflect egg bactericide capacity (Valenti et al., 1983; Valenti et al., 1985), maternal inflammation and infection state (Horrocks et al., 2011) or egg potential for metal ions detoxification (Pohanka et al., 2012).

Contrary to previous observations in feral pigeons (Jacquin et al., 2013), darker females did not transfer higher amounts of specific antibodies than paler females. Mother melanin-based plumage colouration had few effects on early maternal transfer of immune components; nonetheless, it significantly shaped the effects of trace metals exposure on lysozyme concentration. Among birds non-exposed to zinc, lysozyme concentration increased with mother eumelanin level, suggesting that darker females transfer more lysozymes into their eggs than do paler females. However, we found an opposite correlation among zinc-exposed birds (i.e. lysozyme concentration decreased with mother eumelanin level). This result suggests a beneficial effect of zinc on paler females. We may think that zinc is more available for paler birds because darker ones transfer it into their feathers (Chatelain et al., 2014). As a consequence, darker females may transfer higher amounts of zinc into their feathers and therefore benefit less from zinc supplementation.

Altogether, our results demonstrate negative and positive effects of lead and zinc respectively on early maternal transfer of immune components that may explain trace metals effects on birds' reproduction observed *in natura* (Eeva and Lehikoinen, 1996; Eeva et al., 2009; Sens et al., 2003). Because early maternal effects may greatly affect birds' reproductive success and consequently population functioning and dynamics, our study stresses the need to better understand the effects of trace metals on maternal investments. Future studies should investigate the biological significance of decreased or increased transfers of immune components, for instance in juveniles growth, survival and immunity. Moreover, our results suggest that mother eumelanin level increased lysozyme transfer but diminish the beneficial effects of zinc exposure, maybe because of zinc transfer into the feathers. Our study points out the need to investigate the costs and benefits of harbouring highly melanic plumage according to trace metals environmental concentrations.

METHODS

Subjects and Housing

Free-living feral pigeons (C*olumba livia*) were caught in February and March 2014 in several pigeons flocks within the Parisian agglomeration. A sample of 144 pigeons was chosen in such a way as to best equilibrate sex-ratio (72 males and 72 females sexed using discriminant function analysis; Dechaume-Moncharmont et al., 2011) and eumelanin-based plumage coloration

degree; the plumage eumelanic coloration was estimated according to the method described by (Chatelain et al., 2014). Pigeons were kept in 12 outdoor aviaries (3.10 m x 2.00 m x 2.40 m) at the CEREEP field station (Centre d'Ecologie Expérimentale et Prédictive-Ecotron Ile-de-France, UMS 3194, Ecole Normale Supérieure, Saint-Pierre-lès-Nemours, France). They were evenly distributed among aviaries according to their gender, flock and eumelanic plumage colouration intensity in such a way there was no confounding effects between aviaries and these variables (gender: 6 males and 6 females per aviary, flock: $Chi^2=202.19$, df=176, P=0.085, and plumage colouration intensity: $F_{1,144}=0.13$, P=0.721). They were fed *ad libitum* with a mix of maize, wheat and peas. The aviaries were enriched with a bowl of water used for bathing and with branches as perches. Birds were individually identified with a numbered plastic ring. At the end of the experiment (i.e. after 9 months of captivity), birds were released back to the wild at their site of capture.

Treatments

The aviaries were randomly assigned to one of the 4 following metal exposure treatments: exposed to lead only (*lead* group; 10ppm lead acetate, Sigma-Aldrich), exposed to zinc only (*zinc* group; 100ppm zinc sulphate, Prolabo), exposed to both lead and zinc (*lead+zinc* group; 10ppm lead acetate and 100ppm zinc sulphate) or control (*control* group; tap water with none added-metal). Consequently, there were 3 aviaries with 12 pigeons each (36 pigeons in total) per treatment. Metals were diluted in tap water. We chose these concentrations based on both lead blood concentrations measured in urban birds (ranging from 0,053 to 0,264ppm; Roux and Marra, 2007), the gastrointestinal absorption rate of lead in zebra finches (<10%) calculated from Dauwe et al. (2002) and previous supplementation experiments in feral pigeons (Chatelain et al. unpublished). Drinking troughs and baths were filled with the corresponding treated water every other day.

A similar supplementation protocol was used in a previous experiment and its efficiency was validated by measuring lead and zinc concentrations in birds' blood and feathers (Chatelain et al. unpublished). Metals concentrations to which the birds were exposed were 10 times lower than the ones used in this study. Hence, we are confident that metals added to water were indeed ingested by the birds.

Reproduction

Two weeks after the start of the metals treatments, six nest boxes per aviary were opened to allow birds to mate and breed. Feral pigeons commonly produce two-egg-clutches, once to 6 times a year. For each clutch, we randomly collected one of the two eggs and we kept them frozen at -20°C until analysis.

Measurement of specific antibodies transfer

To estimate maternal transfer of specific antibodies into the egg, 20 days after the start of the metal treatments, we subcutaneously injected females (n=72) with 50µg of a Keyhole Limpet Hemocyanin solution (Hemocyanin from *Megathura crenulata*, Sigma Aldrich). Then, we took a blood sample the day the mother laid its first egg, centrifuged it and kept the plasma frozen until analyses. Collected eggs were dissected and eggshell, yolk and albumen were separated one from another. Once unfrozen, the yolk was blend, diluted 1:1 in phosphate buffered saline and homogenized for 1 min with a vortex. Chloroform was then added 1:1 and homogenized for 1 min with a vortex. After centrifugation (6 min at 10 000 g), the supernatant was used for antibodies assays (Jacquin et al., 2013).

Anti-KLH antibody concentrations in mother plasma and egg yolk extractions were measured using a sandwich enzyme-linked immunosorbent assay (ELISA), following the method described by Jacquin et al. (2013).

Measurement of lysozyme transfer

Albumens were unfrozen and homogenized with a vortex. Each well of a 96-well microplate was filled with 9.5µL of albumen or lysozyme standard (lysozyme from chicken egg white, L6876, Sigma-Aldrich, St-Louis, MO, USA). The standards were prepared in phosphate buffered saline (9 $g.L^{-1}$, pH 6.3) for a standard curve ranging from 12.5 to 200 $\mu g.mL^{-1}$ (4°C). 250 µL of micrococcus solution (*Micrococcus lysodeikticus* ATCC No. 4698, M3770, Sigma-Aldrich, St-Louis, MO, USA; DO_{450nm}=1) were added in all the wells and the microplate was left to incubate for 10min at 26°C. Absorbance at 450nm was recorded (t = 10).

Measurement of ovotransferrin transfer

Ovotransferrin transfer was measured following an adapted protocol from Horrocks et al. (2011) and Shawkey et al. (2008). Albumens were defrozen and homogenized with a vortex.

Each well of a 96-well microplate was filled with 24µL of albumen or ovotransferrin standard (conalbumin from chicken egg white, C0755, Sigma-Aldrich, St-Louis, MO, USA). The standards were prepared in reagent one (300mM Tris, 150 mM sodium hydrogen carbonate, 4.2g.L^{-1} Triton X-100, pH=8.4) for a standard curve ranging from 1 to 80 mg.mL^{-1}. 150µL of reagent one containing a 1:32 dilution of iron-standard solution (2000mg.L^{-1}) was added in all the wells. The plate was shaken for 10s and incubated for 5min at 37°C. Following incubation, initial absorbance was recorded at 570nm and 660nm. 25µL of reagent two (50mM Tris, 32.6mM L-ascorbic acid, 10mM Ferrozine, pH=4) was added to each well. The plate was shaken again for 10s and incubated for 5min at 37°C. Finally, 25µL of reagent three (600mM citric acid, 25.6mM Thioura) was added to each well. The plate was shaken for 3s and absorbance was recorded a first time (t0) and a second time after 6min (t6).

First, we corrected for initial differences in absorbance values by subtracting well-specific initial absorbance at 570 and 660nm from the t0 and t6 read at the corresponding wavelength. Then, we determined the difference in absorbance: $\Delta A = A_{570\text{-}660}(t6) - A_{570\text{-}660}(t0)$.

Statistical analyses

A linear mixed-effects model was performed with log-transformed anti-KLH antibodies level in egg as the dependent variable and exposure to zinc, exposure to lead, mother eumelanin level, log-transformed mother anti-KLH antibodies level and their interactions as the explanatory variables. Aviary number and mother identity were added as random factors.

We performed a linear mixed-effects model with lysozyme or ovotransferrin concentration in egg as the dependent variable and exposure to zinc, exposure to lead, mother eumelanin level and their interactions as the explanatory variables. Aviary number and mother identity were added as random factors.

Statistical analyses were performed using R software (version 3.0.2). We retained final models using the AIC.

ACKNOWLEDGMENTS

All experiments were carried out in strict accordance with the recommendations of the "European Convention for the Protection of vertebrate Animals used for Experimental and Other Scientific Purposes" and were conducted under the authorizations of the "Ministère de l'éducation

nationale, de l'enseignement supérieur et de la recherche" (authorization N_00093.02) and the "Direction Départementale des Services Vétérinaires de Seine et-Marne" (authorization N_ 77-05). We thank the "Mairie de Paris" (Thomas Charachon) for allowing the capture of birds and the Centre de Recherche en Ecologie Expérimentale et Prédictive (CEREEP) which provided logistic support for the field work of this study. We are very thankful to S. Béniguel, B. Trouvé, E. Couture, J. Duru, N. Chaumont, S. Perret and B. Decencière for their help all along the field work.

FUNDINGS

This work was supported by the "Sorbonne Universités" ; the "Université Pierre & Marie Curie" ; the "Université Parie Est Créteil" ; the "Université Paris Diderot" ; the "Centre National de la Recherche Scientifique" ; the "Institut National de la Recherche Agronomique" ; and the "Institut de Recherche pour le Développement".

AUTHORS CONTRIBUTION

MC participated in the design of the study, carried out the field and lab work, analysed the data and wrote the manuscript; JG and AF participated in the design of the study, helped in data analysis and draft the manuscript. CH participated in the lab work. All authors gave their final approval for publication.

The authors have declared that no competing interest exists.

REFERENCES

Agusa, T., Matsumoto, T., Ikemoto, T., Anan, Y., Kubota, R., Yasunaga, G., Kunito, T., Tanabe, S., Ogi, H. and Shibata, Y. (2005). Body distribution of trace elements in black-tailed gulls from Rishiri Island, Japan: Age-dependent accumulation and transfer to feathers and eggs. *Environ. Toxicol. Chem.* 24, 2107–2120.

Azimi, S., Ludwig, A., Thévenot, D. R. and Colin, J.-L. (2003). Trace metal determination in total atmospheric deposition in rural and urban areas. *Sci. Total Environ.* 308, 247–256.

Azimi, S., Rocher, V., Muller, M., Moilleron, R. and Thevenot, D. R. (2005). Sources, distribution and variability of hydrocarbons and metals in atmospheric deposition in an urban area (Paris, France). *Sci. Total Environ.* 337, 223–239.

Cerklewski, F. L. and Forbes, R. M. (1976). Influence of dietary zinc on lead toxicity in the rat. *J. Nutr.* 106, 689–696.

Chatelain, M., Gasparini, J., Jacquin, L. and Frantz, A. (2014). The adaptive function of melanin-based plumage coloration to trace metals. *Biol. Lett.* 10, 20140164–20140164.

Dauwe, T., Janssens, E., Pinxten, R. and Eens, M. (2005). The reproductive success and quality of blue tits (*Parus caeruleus*) in a heavy metal pollution gradient. *Environ. Pollut.* 136, 243–251.

Dauwe, L. Bervoets, R. Blust, M. Ee, T. (2002). Tissue levels of lead in experimentally exposed zebra finches (*Taeniopygia guttata*) with particular attention on the use of feathers as biomonitors. *Arch. Environ. Contam. Toxicol.* 42, 88–92.

Dechaume-Moncharmont, F.-X., Monceau, K. and Cezilly, F. (2011). Sexing birds using discriminant function analysis: A Critical Appraisal. *The Auk* 128, 78–86.

Dibner, J. J., Knight, C. D., Kitchell, M. L., Atwell, C. A., Downs, A. C. and Ivey, F. J. (1998). Early feeding and development of the immune system in neonatal poultry1. *J. Appl. Poult. Res.* 7, 425–436.

Ducrest, A., Keller, L. and Roulin, A. (2008). Pleiotropy in the melanocortin system, coloration and behavioural syndromes. *Trends Ecol. Evol.* 23, 502–510.

Eeva, T. and Lehikoinen, E. (1996). Growth and mortality of nestling great tits (*Parus major*) and pied flycatchers (*Ficedula hypoleuca*) in a heavy metal pollution gradient. *Oecologia* 108, 631–639.

Eeva, T., Ahola, M. and Lehikoinen, E. (2009). Breeding performance of blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*) in a heavy metal polluted area. *Environ. Pollut.* 157, 3126–3131.

El-Gazzar, R. M., Finelli, V. N., Boiano, J. and Petering, H. G. (1978). Influence of dietary zinc on lead toxicity in rats. *Toxicol. Lett.* 1, 227–234.

Gasparini, J., McCoy, K. D., Staszewski, V., Haussy, C. and Boulinier, T. (2006). Dynamics of anti-*Borrelia* antibodies in black-legged kittiwake (*Rissa tridactyla*) chicks suggest a maternal educational effect. *Can. J. Zool.* 84, 623–627.

Gasparini, J., Boulinier, T., Gill, V. A., Gil, D., Hatch, S. A. and Roulin, A. (2007). Food availability affects the maternal transfer of androgens and antibodies into eggs of a colonial seabird. *J. Evol. Biol.* 20, 874–880.

Gasparini, J., Jacquin, L., Laroucau, K., Vorimore, F., Aubry, E., Castrec-Rouëlle, M. and Frantz, A. (2014). Relationships between metals exposure and epidemiological parameters of two pathogens in urban pigeons. *Bull. Environ. Contam. Toxicol.* 92, 208–212.

Godwin, H. A. (2001). The biological chemistry of lead. *Curr. Opin. Chem. Biol.* 5, 223–227.

Hargitai, R., Prechl, J. and TöRöK, J. (2006). Maternal immunoglobulin concentration in Collared Flycatcher (*Ficedula albicollis*) eggs in relation to parental quality and laying order. *Funct. Ecol.* 20, 829–838.

Hasselquist, D. and Nilsson, J.-A. (2009). Maternal transfer of antibodies in vertebrates: trans-generational effects on offspring immunity. *Philos. Trans. R. Soc. B Biol. Sci.* 364, 51–60.

Heller, E. D., Leitner, H., Drabkin, N. and Melamed, D. (1990). Passive immunisation of chicks against *Escherichia coli*. *Avian Pathol.* 19, 345–354.

Horrocks, N. P. C., Irene Tieleman, B. and Matson, K. D. (2011). A simple assay for measurement of ovotransferrin - a marker of inflammation and infection in birds: Ovotransferrin assay for plasma from wild birds. *Methods Ecol. Evol.* 2, 518–526.

Ismail, A., Jacquin, L., Haussy, C., Perret, S. and Gasparini, J. (2015). Food availability modulates the effects of maternal antibodies on growth and immunity in young feral pigeons. *J. Avian Biol.* n/a–n/a.

Jacquin, L., Haussy, C., Bertin, C., Laroucau, K. and Gasparini, J. (2013). Darker female pigeons transmit more specific antibodies to their eggs than do paler ones. *Biol. J. Linn. Soc.* 108, 647–657.

Jarup, L. (2003). Hazards of heavy metal contamination. *Br. Med. Bull.* 68, 167–182.

Maas, S., Scheifler, R., Benslama, M., Crini, N., Lucot, E., Brahmia, Z., Benyacoub, S. and Giraudoux, P. (2010). Spatial distribution of heavy metal concentrations in urban, suburban and agricultural soils in a Mediterranean city of Algeria. *Environ. Pollut.* 158, 2294–2301.

Masschalck, B. and Michiels, C. W. (2003). Antimicrobial properties of lysozyme in relation to foodborne vegetative bacteria. *Crit. Rev. Microbiol.* 29, 191–214.

Mauck, R. A., Matson, K. D., Philipsborn, J. and Ricklefs, R. E. (2005). Increase in the constitutive innate humoral immune system in Leach's storm-petrel (*Oceanodroma leucorhoa*) chicks is negatively correlated with growth rate. *Funct. Ecol.* 19, 1001–1007.

Patrick, L. (2006). Lead toxicity, a review of the literature. Part 1: Exposure, evaluation, and treatment. *Altern. Med. Rev. J. Clin. Ther.* 11, 2–22.

Pellegrini, A., Thomas, U., von Fellenberg, R. and Wild, P. (1992). Bactericidal activities of lysozyme and aprotinin against Gram-negative and Gram-positive bacteria related to their basic character. *J. Appl. Bacteriol.* 72, 180–187.

Pihlaja, M., Siitari, H. and Alatalo, R. V. (2006). Maternal antibodies in a wild altricial bird: effects on offspring immunity, growth and survival: Maternal antibodies in an altricial bird. *J. Anim. Ecol.* 75, 1154–1164.

Pohanka, M., Hubalek, J., Beklova, M., Vrba, R. and Kizek, R. (2012). Metallomics study of lead-protein interactions in albumen by electrochemical and electrophoretic methods. *Int J Electrochem Sci* 7, 943–964.

Prasad, A. S. (1998). Zinc and immunity. *Mol. Cell. Biochem.* 188, 63–69.

Prasad, A. S. (2009). Zinc: role in immunity, oxidative stress and chronic inflammation: *Curr. Opin. Clin. Nutr. Metab. Care* 12, 646–652.

Redig, P. T., Lawler, E. M., Schwartz, S., Dunnette, J. L., Stephenson, B. and Duke, G. E. (1991). Effects of chronic exposure to sublethal concentrations of lead acetate on heme synthesis and immune function in red-tailed hawks. *Arch. Environ. Contam. Toxicol.* 21, 72–77.

Roux, K. E. and Marra, P. P. (2007). The presence and impact of environmental lead in passerine birds along an urban to rural land use gradient. *Arch. Environ. Contam. Toxicol.* 53, 261–268.

Saino, N., Dall'Ara, P., Martinelli, R. and Møller, A. P. (2002a). Early maternal effects and antibacterial immune factors in the eggs, nestlings and adults of the barn swallow. *J. Evol. Biol.* 15, 735–743.

Saino, N., Ferrari, R. P., Martinelli, R., Romano, M., Rubolini, D. and Moller, A. P. (2002b). Early maternal effects mediated by immunity depend on sexual ornamentation of the male partner. *Proc. R. Soc. B Biol. Sci.* 269, 1005–1009.

Sens, E. J., Dauwe, T., Pinxten, R. and Eens, M. (2003). Breeding performance of great tits (*Parus major*) along a gradient of heavy metal pollution. *Environ. Toxicol. Chem.* 22, 1140–1145.

Shawkey, M. D., Kosciuch, K. L., Liu, M., Rohwer, F. C., Loos, E. R., Wang, J. M. and Beissinger, S. R. (2008). Do birds differentially distribute antimicrobial proteins within clutches of eggs? *Behav. Ecol.* 19, 920–927.

Smith, M. O. (2003). Effects of different levels of zinc on the performance and immunocompetence of broilers under heat stress. *Poult. Sci.* 82, 1580–1588.

Snoeijs, T., Dauwe, T., Pinxten, R., Vandesande, F. and Eens, M. (2004). Heavy metal exposure affects the humoral immune response in a free-living small songbird, the great tit (*Parus major*). *Arch. Environ. Contam. Toxicol.* 46.

Snoeijs, T., Dauwe, T., Pinxten, R., Darras, V., Arckens, L. and Eens, M. (2005). The combined effect of lead exposure and high or low dietary calcium on health and immunocompetence in the zebra finch. *Environ. Pollut.* 134, 123–132.

Trust, K. A., Miller, M. W., Ringelman, J. K. and Orme, I. M. (1990). Effects of ingested lead on antibody production in mallards (*Anas platyrhynchos*). *J. Wildl. Dis.* 26, 316–322.

Valenti, P., Antonini, G., Von Hunolstein, C., Visca, P., Orsi, N. and Antonini, E. (1983). Studies of the antimicrobial activity of ovotransferrin. *Int. J. Tissue React.* 5, 97–105.

Valenti, P., Visca, P., Antonini, G. and Orsi, N. (1985). Antifungal activity of ovotransferrin towards genus Candida. *Mycopathologia* 89, 169–175.

Chapitre 5 :

Métaux traces et communauté bactérienne du plumage

Introduction

Le plumage est colonisé par de nombreuses bactéries et champignons, qui constituent le microbiote du plumage. Néanmoins, relativement peu d'études s'y sont intéressées et nos connaissances sur le rôle de ces organismes sont réduites. Le plumage renfermerait un écosystème microbien dont l'équilibre déterminerait, entre autres, la qualité du plumage. Cet équilibre dépend de nombreux facteurs intrinsèques (Hildebrand et al., 2013; Mueller et al., 2006) ou extérieurs à l'hôte (Burkholder et al., 2008; Dotterud et al., 2008; Ruiz-de-Castañeda et al., 2011). Notamment, il est susceptible d'être affecté par l'exposition aux métaux traces du fait d'effets toxiques ou bénéfiques directs des métaux accumulés sur le plumage (Frantz et al., 2012) sur les bactéries (e.g. l'ingestion de plomb modifie l'abondance relative des bactéries de l'intestin chez la souris Breton et al., 2013). De plus, les métaux traces ingérés altèrent la physiologie de l'oiseau, et notamment sa capacité à lutter contre des infections (Chatelain et al. soumis; Snoeijs et al., 2005, 2004) ; ceci pourrait par conséquent réduire le contrôle qu'ont les oiseaux sur la communauté bactérienne de leur plumage via les sécrétions uropygiennes antimicrobiennes qu'ils appliquent lors du lissage des plumes (Image 3), comportement par ailleurs connu pour être coûteux (Jacob et al., 2014; Moreno-Rueda, 2010; Piault et al., 2008). Encore une fois, la coloration mélanique du plumage est susceptible de moduler les effets indirects des métaux traces (i.e. leurs effets sur la physiologie des oiseaux) sur la communauté bactérienne du plumage. Par ailleurs, elle pourrait directement affecter la communauté bactérienne du plumage puisque certaines études montrent des différences de résistance des plumes à la dégradation en fonction de leur degré de mélanisation (Goldstein et al., 2004; Gunderson et al., 2008).

Image 3. Comportement de lissage des plumes (« preening » chez un pigeon biset.

Méthodes

Au cours de l'expérience réalisée en 2013 (étude expérimentale 1), la composition (i.e. identification des OTUs) et la richesse (i.e. nombre d'OTUs) de la communauté bactérienne a été mesurée à partir de plumes prélevées sur le dos de chaque adulte. De plus, l'abondance bactérienne a été estimée par culture sur lames gélosées (Image 4). Par ailleurs, le comportement des oiseaux a été observé et le temps passé au lissage des plumes a été chronométré.

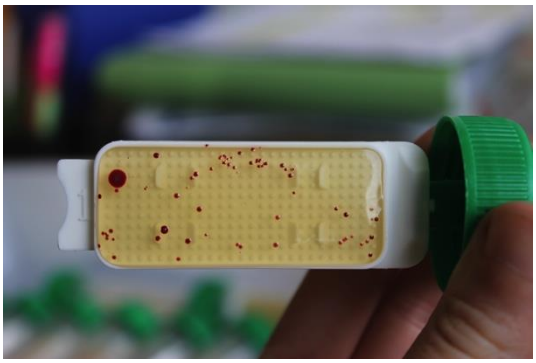


Image 4. Lame gélosée après avoir été appliquée sur le plumage d'un pigeon et incubée pendant 24h ; chaque point rouge correspond à une colonie bactérienne.

Conclusion

Pour la première fois, notre étude met en évidence des effets du plomb et du zinc sur la communauté bactérienne du plumage. L'exposition au plomb semble entraîner les plus grandes modifications, à la fois en changeant la composition des communautés bactériennes, mais aussi en diminuant la richesse bactérienne et en diminuant le temps alloué par les oiseaux à se lisser les plumes. L'exposition au zinc montre elle aussi des effets toxiques en diminuant l'abondance bactérienne. Ces différences de composition des communautés bactériennes sont susceptibles d'entrainer des différences de qualité du plumage, notamment au niveau de leur capacité à garder la chaleur, ainsi que de leur coloration structurelle (Gunderson et al., 2009; Shawkey et al., 2007). Dans ce sens, nous avons montré chez le pigeon biset que la diminution de l'abondance bactérienne augmentait la qualité du plumage (i.e. son état de dégradation, évalué visuellement), alors que son augmentation était associée à une coloration plus claire (i.e. plus forte réflectance) des plumes iridescentes de la gorge (Leclaire et al., 2014, voir Annexe). De façon cohérente, nous avons également mis en évidence une augmentation de la réflectance des plumes iridescentes de la gorge chez les individus exposés au plomb (étude expérimentale 1 ; Figure 8 ; résultats n'ayant pas fait l'objet d'un manuscrit).

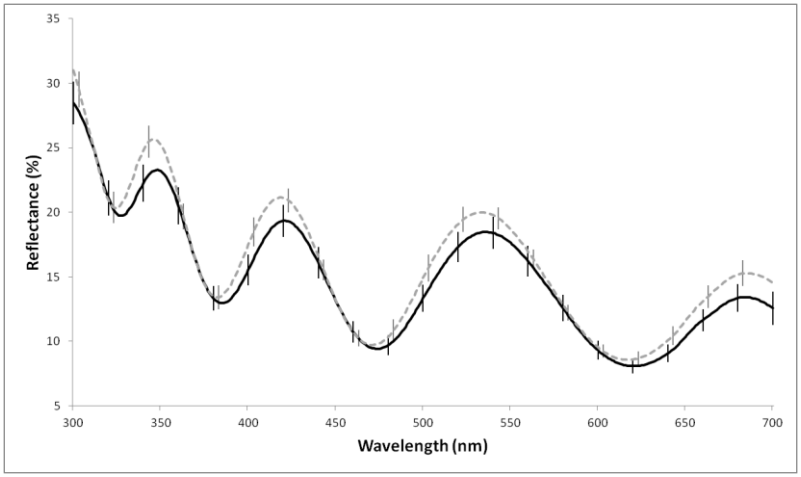


Figure 8. Spectre de réflectance (moyenne ± erreur standard) des plumes iridescentes vertes de la gorge de pigeons bisets exposés au plomb (groupes plomb et zinc+plomb ; ligne pointillée) ou non (groupes contrôle et zinc ; ligne pleine).

Afin de mieux comprendre comment les métaux traces peuvent affecter la qualité du plumage, il apparaît nécessaire d'identifier les espèces bactériennes présentes. Ceci nous permettrait de mettre en évidence d'éventuels mécanismes de sélection d'espèces résistantes aux métaux traces, suggérée par la diminution de la richesse bactérienne en présence de plomb. De plus, nous pourrions ainsi mesurer l'abondance relative des espèces pathogènes (e.g. kératinophages) et commensales ; afin de connaître les effets des métaux traces sur les bactéries et d'identifier les effets indirects des métaux traces sur l'hôte (i.e. via la communauté bactérienne du plumage), qu'ils soient négatifs ou positifs. De plus, il serait intéressant de mener une étude *in vitro* testant les effets d'une exposition aux métaux traces sur les communautés bactériennes de plumes préalablement prélevées, afin de distinguer les effets directs des métaux sur les bactéries, des effets indirects résultant d'une modification de la physiologie de l'hôte. En effet, bien que notre étude montre un effet négatif de l'exposition au plomb sur le comportement de lissage des plumes, cela peut résulter du coût physiologique d'une telle exposition, qui ne permettrait pas aux oiseaux d'investir dans ce comportement, ou de l'ajustement par les oiseaux du temps investi dans ce comportement en réponse à la modification de la communauté bactérienne de leur plumage (Leclaire et al., 2014).

La qualité du plumage est un paramètre essentiel chez la plus part des espèces d'oiseaux, influençant à la fois leur capacité de thermorégulation et le choix de partenaires sexuels. Par exemple, chez le pigeon biset, la qualité et la couleur des plumes sont deux des critères utilisés lors de la sélection d'un partenaire (Johnston and Janiga, 1995). Aussi, tout paramètre (e.g. métaux traces) modifiant la communauté bactérienne du plumage est susceptible d'influencer les traits d'histoire de vie des individus (e.g. résistance au froid, probabilité de reproduction).

Ce chapitre prend la forme d'un article soumis dans The Condor.

Experimental exposure to trace metals affects plumage bacterial community in the feral pigeon

Chatelain M[1*], Frantz A[1], Gasparini J[1] and Leclaire S[1,2]

[1] Sorbonne Universités, UPMC Univ Paris 06, UPEC, Paris 7, CNRS, INRA, IRD, Institut d'Ecologie et des Sciences de l'Environnement de Paris, F-75005, Paris, France

[2] Centre d'Ecologie Fonctionnelle et Evolutive, UMR 5175, CNRS, 1919 route de Mende, 34293 Montpellier, France

*Corresponding author: Marion Chatelain, Address : Université Pierre et Marie Curie 7 quai St Bernard 75005 Paris France, E-mail : marion.chatelain@upmc.fr, Tel: +33144275204

ABSTRACT

Bacteria are fundamental associates of animals, and being beneficial or detrimental to their host can ultimately shape their host fitness. Host-associated bacteria are highly influenced by various environmental factors. In birds, trace metals emitted by anthropogenic activities are known to accumulate onto the plumage, and once ingested, to induce negative or positive effects on animal physiology. Trace metals may thus alter plumage bacterial community directly or indirectly by decreasing bird condition and shaping bird investment in antibacterial defences. Although trace metals are current major environmental issues in urban habitats, their effect on feather bacterial community has however never been investigated. Here, we supplemented feral pigeons (*Columba livia*), an emblematic urban species, with zinc and/or lead in drinking and bath water. Lead and zinc were found to shape the plumage bacterial community composition, richness and density, and altered bird preening behaviour. Our results are the first to demonstrate the effects of common urban trace metals on plumage bacterial community. Further studies are now needed to investigate how this change modulates the life history traits that are known to depend on plumage bacterial community.

Key words: urban ecology, birds, bacteria

INTRODUCTION

Bacteria colonize numerous habitats and more particularly animal body parts that are in direct contact with the surrounding such as skin, feathers or fur, and the digestive tract. The normal flora, also called the microbiota, lives in symbiosis with its host, fulfilling essential functions for the host metabolism, such as degrading cellulose, synthesizing vitamins (Hill 1997) and inhibiting the proliferation of pathogens (Balcázar et al. 2007, Oh et al. 2006, Olsson et al. 1992). The microbiota is influenced by numerous interconnected factors, including the host behaviour, genotype and physiology (Frank et al. 2011, Hildebrand et al. 2013, Leclaire et al. 2014a, Mueller et al. 2006, Rosenthal et al. 2011), as well as factors of its surrounding environment (Burkholder et al. 2008, Dotterud et al. 2008, Ruiz-de-Castañeda et al. 2011).

Compared to rural environments, urban areas are highly polluted by trace metals emitted by anthropogenic activities, (Azimi et al. 2005, Kekkonen et al. 2012, Roux and Marra 2007, Scheifler et al. 2006). Some trace metals, such as lead, cadmium, zinc, copper, chrome and nickel, are known to have toxic effects on environmental microbial communities (Babich and Stotzky 1978, Giller et al. 1998). In contrast, although trace metals can be naturally ingested or inhaled by animals, and deposited on integuments, their effects on animal bacterial community have been poorly investigated (but see Breton et al. 2013, Hojberg et al. 2005, Liu et al. 2014, Vahjen et al. 2010).

Like other integuments, the plumage is highly colonized by bacteria (Burtt and Ichida 1999, Muza et al. 2000, Shawkey and Hill 2004, Whitaker et al. 2005). The plumage, as well as uropygial gland secretions spread onto it, can accumulate metals (Frantz et al. 2012, Pilastro et al. 1993), which can affect plumage bacterial community by direct contact. Ingested metals also circulate in the bloodstream, accumulate in organs and bones (Dauwe, Bervoets et al. 2002, Kekkonen et al. 2012, Pattee 1984, Reid et al. 2012, Scheifler et al. 2006), and may induce noxious effects on bird physiology (Dauwe et al. 2005, Eeva et al. 2009, Redig et al. 1991, Snoeijs et al. 2004). However, some metals are also essential nutrients when absorbed in low concentrations (Prasad 1998). Trace metals may therefore shape bird investment in the costly behavioural (e.g. Preening) and physiological mechanisms they use to control plumage bacterial load and composition (Leclaire et al. 2014b, Moreno-Rueda 2010, Piault et al. 2008). Some feather bacteria alter feather structure (Burtt and Ichida 1999, Shawkey et al. 2007) and may affect sexual signalization such as feather coloration or plumage condition (Gunderson et al. 2009, Leclaire et al. 2014b, Shawkey et al. 2007). In addition, feather bacteria can have strong impact on bird immune system (Leclaire et al. 2015) and reproduction (Jacob et al. 2015). Whatever the mechanisms by which metals affect feather

bacteria, further studies are clearly needed to evaluate the effects of trace metals on feather bacteria.

The feral pigeon (*Columba livia*) is an urban bird living in high density and proximity which may promote an elevated plumage bacteria transmission between individuals (reviewed in Archie and Theis 2011). Mate choice is crucial for this species which mate for life (Johnston and Janiga 1995). Feather condition and colouration, which depend on bacterial load (Leclaire et al. 2014b), are two of the main criteria used in mate choice in this species (Johnston and Janiga 1995). Consequently, trace metals may have a great impact on pigeon reproduction, through their effects on plumage bacterial load and composition. Here, we investigated the effects of an experimental exposure to lead and/or zinc on pigeon plumage bacteria and preening behavior, an index of birds' investment in controlling the bacterial community of their plumage.

METHODS

Subjects and housing

Ninety six (48 males and 48 females) free-living adult Feral pigeons (*Columba livia*) were caught during winter 2013 (February/March) in the Parisian agglomeration. The birds were immediately transferred in 8 outdoor aviaries (2.20 m x 2.20 m) at the CEREEP field station (Centre d'Ecologie Expérimentale et Prédictive-Ecotron Ile-de-France, UMS 3194, Ecole Normale Supérieure, Saint-Pierre-lès-Nemours, France). Birds were fed *ad libitum* with a mix of maize, wheat and peas. The aviaries were enriched with a bowl of water used for bathing and with branches as perches. Birds were individually identified with a numbered plastic ring. Birds were genetically sexed following the protocol described by (Griffiths et al. 1998). Before onset of treatment, birds were kept 2 to 7 weeks for acclimation. At the end of the experiment, all birds were released back to the wild at their site of capture.

Measurement of plumage colouration

At their capture, birds were first categorised as eumelanic (grey to black pigmented) or pheomelanic (red pigmented) which define their melanin type. Then, eumelanic birds were individually photographed in order to measure their eumelanin level. Eumelanin level was calculated as the percentage of black on the wing surface of birds (number of black pixels/number of white pixels x 100) using the Gimp image retouching and editing software which is a reliable and repeatable estimation of melanin concentration (Jacquin et al. 2011).

Because of the small amount of pheomelanic birds (14 over 96), the measure of a pheomelanin level was not relevant.

Treatments

Two weeks before onset of treatment, aviaries were divided into 4 metal-exposure treatments; this means there were 2 aviaries with 12 pigeons each per treatment (24 pigeons in total per treatment). For each treatment, the two aviaries were purposely spatially separated from one another (Table 1). Aviaries were in direct contact along a linear transect and numbered from 1 to 8 (Table 1). Side-by-side aviaries were separated by wire mesh, Treatments consisted of water supplemented with lead (*lead* group; 1ppm lead acetate; aviaries 1 and 5), zinc (*zinc* group; 10 ppm zinc sulphate; aviaries 2 and 7), lead and zinc (*lead+zinc* group; 1ppm lead acetate and 10ppm zinc sulphate; aviaries 4 and 8), or control (*control group;* tap water with no metal added; aviaries 3 and 6). We equally distributed pigeons among aviaries according to sex (6 females and 6 males per aviaries) and to their place of capture (Chi^2=71.09, df=70, P=0.441). Drinking troughs and baths were filled with the corresponding treated water every other day. Our supplementation treatments were validated by measuring lead and zinc concentrations in bird blood and feathers. Blood was sampled 10 weeks after the start of the experiment. Moreover, the fifth secondary remige of each bird was removed a first time and the regrowth was used for metal measurements. Both blood and feathers were digested using a previously described protocol (Chatelain et al. 2014) and lead and zinc concentrations were measured by mass spectrometry (ICP-MS) and by optic emission spectrometry (ICP-OES) respectively. Lead and zinc blood concentrations were higher among birds exposed to lead *(lead* and *lead+zinc* groups; $F_{1,45}$=4,47, P=0.040) and birds exposed to zinc (*zinc* and *lead+zinc* groups; $F_{1,67}$=5.52, P=0.022) respectively. In the feathers, while lead concentrations were significantly higher among birds exposed to lead *(lead* and *lead+zinc* groups; $F_{1,76}$=19.61, P<0.001), the increase in zinc concentration among birds exposed to zinc (*zinc* and *lead+zinc* groups) was not significant ($F_{1,76}$=2.13, P=0.149). These results ensure that metals added to water was ingested by the birds.

Aviary	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Treatment	*Lead*	*Zinc*	*Control*	*Lead + zinc*	*Lead*	*Control*	*Zinc*	*Lead + zinc*

Tab. 1 Metal-exposure treatments distribution among the 8 aviaries.

Measurement of plumage bacterial load

Plumage bacterial load was measured 20 weeks after onset of treatment. 4 hours after renewing the water of the bowls used for bathing, 10 birds (5 males and 5 females) were sampled in each treatment. Each sampled bird was caught with a net that had previously been sprayed with 70% ethanol. Then, a whole flora agar slide (Hygialim, 3026091, Plate Count Agar + triphenyltetrazolium chloride+Neutralizing) was put flat on the back of the bird for 10 seconds. The slides were then incubated at 37°C for 24h. Feather bacterial load was expressed as the number of bacterial colonies per slide.

Molecular analysis of plumage bacterial communities

Fifteen weeks after the start of the experiment, 91 adults were caught with a net that had previously been sprayed with 70% ethanol. After washing its hands with alcohol, the experimenter cut a clump (10 feathers on average) of back feathers with sterilized scissors and pliers, avoiding the feathers to be in direct contact with the surrounding. The feathers were immediately placed in sterile 2ml plastic tubes and stored at -20°C until analysis.

We extracted DNA using the Qiagen dneasy® Blood and Tissue Kit and the standard protocol designed for the purification of total DNA from Gram-positive bacteria (Qiagen, Venlo, Netherlands; July 2006).

To characterize the bacterial communities present in each sample, we performed automated ribosomal intergenic spacer analyses (ARISA; Ranjard et al. 2000). This DNA fingerprinting method is based on the amplification of the internal transcribed spacer (ITS) region lying between the 16S and 23S ribosomal RNA genes in the ribosomal operon. The ITS region is extremely variable, in both sequence and length, for different bacterial species. Therefore, the DNA amplification profile obtained with ARISA allows straightforward estimation of bacterial diversity, avoiding biases inherent in classical culture-based techniques (Ranjard et al. 2000). We amplified the ITS using the FAM (6-carboxyfluorescein)- labeled primer S-D-Bact-1522-b-S-20 (5'-[6FAM] TGCGGCTGG ATCCCCTCCTT-3') and the unlabeled primer L-D-Bact-132- a-A-18 (5'-CCGGGTTTCCCCATTCGG-3') (Ranjard et al. 2000). We performed the PCR amplification in 10 µl mixtures containing 200 µm each deoxynucleotide triphosphate, 0.20 µm each primer, 1.25 units of perfect*taq* DNA polymerase, 1× PCR buffer (5 Prime, gmbh, Hamburg, Germany), and 1 µl DNA extract, using the following protocol: initial denaturation at 94 °C for 3 min, 40 cycles consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, elongation at 72 °C for 1 min, and a final elongation at 72 °C for 10 min. We then mixed 1 µl of the PCR products with 15 µl of highly deionized formamide and 0.2 µl of Genescan 1200 LIZ size standard (Applied Biosystems, Foster City, CA). The mixtures were denatured at

95 °C for 5 min before separation with a 24-capillary 3500XL DNA Analyzer (Applied Biosystems) using POP-7 polymer and the manufacturer's default electrophoresis run settings. Data analysis and genotyping were performed with genemapper software (Applied Biosystems). For each sample, the sequencer produced an ARISA profile in which each peak corresponds to 1 phylotype or operational taxonomic unit (OTU). In the various samples, the sequencer detected ITS fragments ranging in size from 300 to 950 base pairs.

For each individual, we calculated bacterial richness as the number of different otus. Because the probability to detect an OTU is likely affected by the amount of feathers used for DNA extraction, feathers were dried overnight and weighted to the nearest mg after DNA extraction. We estimated bacterial community dissimilarities between individuals using Jaccard distance based on presence/absence of OTUs.

Observations of preening behaviour

An observer blind to the treatment recorded a total of 95 independent behavioural sessions of 5 minutes each. The observer was situated outside of the aviary and waited few minutes before starting her observations in order birds' behaviour not to be influenced by the presence of the observer. Observed birds were randomly chosen but the observer changed treatment between each observation to have a comparable number of observations between each treatment: 25 (21 different individuals), 29 (24 different individuals), 22 (20 different individuals) and 19 (16 different individuals) observations respectively among controls, birds exposed to lead only, birds exposed to zinc only and birds exposed to both lead and zinc. The records were done with the jwatcher software. Among several predefined behaviours, we recorded the time the birds spent preening, a known antibacterial behaviour. During preening, birds spread preen secretion, which have antimicrobial proprieties (Czirják et al. 2013, Ruiz-Rodriguez et al. 2009, Shawkey et al. 2003), onto the plumage. The time pigeons allocate to this costly behaviour (Leclaire et al. 2014b, Moreno-Rueda 2010, Piault et al. 2008) may be an index of bacterial community load (Leclaire et al. 2014b) but may also reflect bird health status. For instance, juvenile apapanes (*Himatione sanguinea*) experimentally infected with *Plasmodium relictum* spent less time preening (Yorinks and Atkinson 2000).

Statistical analyses

Statistical analyses were performed using R (R.3.0.2; R Development Core Team).

To test for the effects of metal exposure on the composition of bacterial communities, we performed a PERMANOVA with 5000 permutations (i.e. Nonparametric multivariate analysis of variance, Adonis function, VEGAN package in R (Oksanen et al. 2007), based on Jaccard distance for OTU presence/absence data. Zinc exposure, lead exposure, sex and their

interactions were included as explanatory variables. Because spatial proximity may influence bacterial community similarity between individuals, we added the aviary as a covariate. Then, we ran similar analyses between each pair of treatment. Finally, we tested the differences of bacterial communities between aviaries among each metal treatment.

To investigate more precisely the effect of spatial proximity on bacterial communities' similarities, we compared a matrix of bacterial Jaccard distances between individuals to a matrix of spatial distances (scored as 0 for individuals inhabiting the same aviary, to 7 for the most distant individuals), considering a matrix of treatment membership (scored as 0 for individuals submitted to the treatment and 1 for individuals of different treatments) using partial mantel test with 5000 permutations.

We graphically represented similarities between individuals using a constrained redundancy analysis (RDA function in R) based on the Jaccard distance matrix.

We also tested plumage bacterial richness using a linear mixed model for Poisson distribution with zinc exposure, lead exposure, sex and their interactions as explanatory variables, and the weight of feathers used for the analysis as a covariate.

To test for the effects of metal exposure on plumage bacterial load and because our sample size was low (n=10 per treatment), we performed Wilcoxon tests. First, we tested for an effect of zinc and for an effect of lead in two different tests; then, we performed Wilcoxon tests between each pairs of treatments to test for an effect of the interaction between zinc and lead exposure.

Finally, we investigated the amount of time birds allocated to preening by performing a generalized linear mixed model for Poisson distribution with zinc exposure, lead exposure, sex and their interactions as explicative variables and birds' identity and aviary as a random effect.

For all analysis, the same models were performed on eumelanic birds only (i.e. excluding pheomelanic pigeons). In these models, plumage eumelanin level and its interaction with the other considered parameters were added as explanatory variables.

RESULTS

The composition of bacterial communities depended on the interaction between lead and zinc exposure ($F_{1,82}$=3.47, P<0.001) and on aviary ($F_{1,82}$=3.91, P<0.001 respectively; Fig. 1). Each pairwise test between metal exposures was significant (Table 2). The composition of bacterial

communities differed significantly between aviaries among each metal treatment but was less dissimilar among *lead* group (Table 3). Moreover, there was a highly significant positive correlation between bacterial distance and spatial distance (r=0.30, P<0.001; Fig. 2).

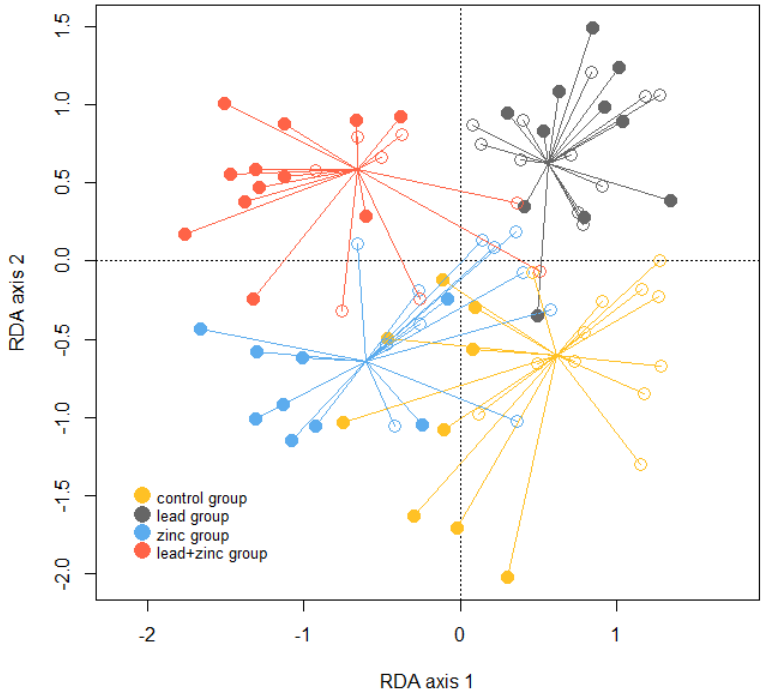


Fig. 1 Constrained redundancy analysis (RDA function in R) on bacterial community dissimilarities (estimated by Jaccard distances) between lead, zinc, lead+zinc and control groups. Both aviaries of a same group are distinguished using filled or empty circles.

	Control	Zinc-exposure	Lead-exposure
Zinc-exposure	$F_{1,40}$=2.74, P=0.002	-	-
Lead-exposure	$F_{1,42}$=3.44, P<0.001	$F_{1,41}$=3.16, P<0.001	-
Zinc and lead-exposure	$F_{1,40}$=4.40, P<0.001	$F_{1,39}$=2.10, P=0.006	$F_{1,41}$=5.13, P<0.001

Tab. 2 PERMANOVAs with 5000 permutations based on Jaccard distance for OTU presence/absence data with the metal exposure as the explicative variable. Bacterial communities' similarities were compared between each pair of metal treatment.

	Differences between aviaries
Zinc-exposure	$F_{1,19}$=3.48, P<0.001
Lead-exposure	$F_{1,21}$=1.70, P=0.018
Zinc and lead-exposure	$F_{1,19}$=4.48, P<0.001
Control	$F_{1,20}$=7.59, P<0.001

Tab. 3 PERMANOVAs with 5000 permutations based on Jaccard distance for OTU presence/absence data with the aviary as the explicative variable. Bacterial communities' similarities were compared between aviaries among each metal treatment.

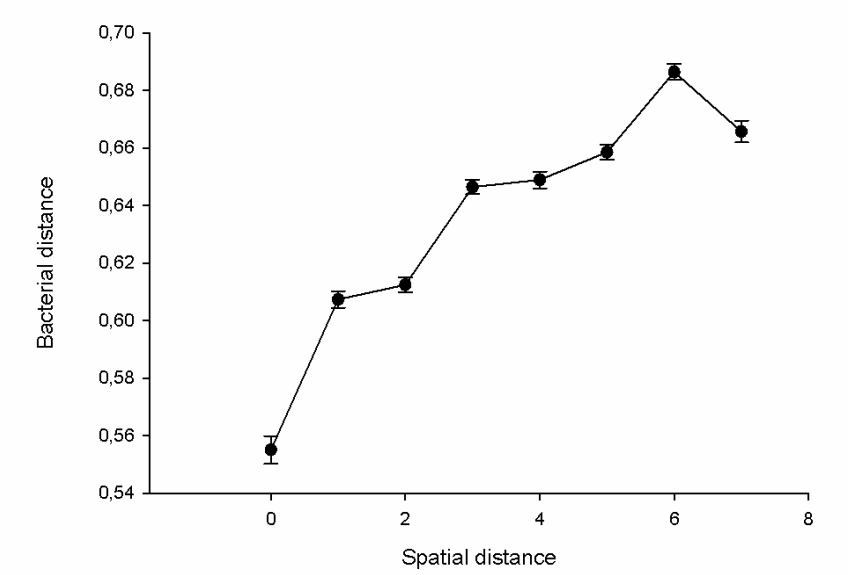


Fig. 2 Mean ± SE plumage bacterial community dissimilarities (estimated by Jaccard distance) in dyads of pigeons according to the spatial distance between them (0 means that the individuals belonged to the same aviary, 1 means that they were in side-by-side aviaries, 2 means that 1 aviary was between them, etc.).

Plumage bacterial richness depended on the interaction between zinc and lead-exposure (Chi^2=9.11, df=80, P=0.003; Fig. 3): *lead* group had lower bacterial richness than c*ontrol* group (Chi^2=5.59, df=42, P=0.018) and *zinc* group (Chi^2=12.66, df=41, P<0.001). Moreover, *lead+zinc* group tended to have a higher plumage bacterial richness than had *zinc* group (Chi^2=3.64, df=39, P=0.057).

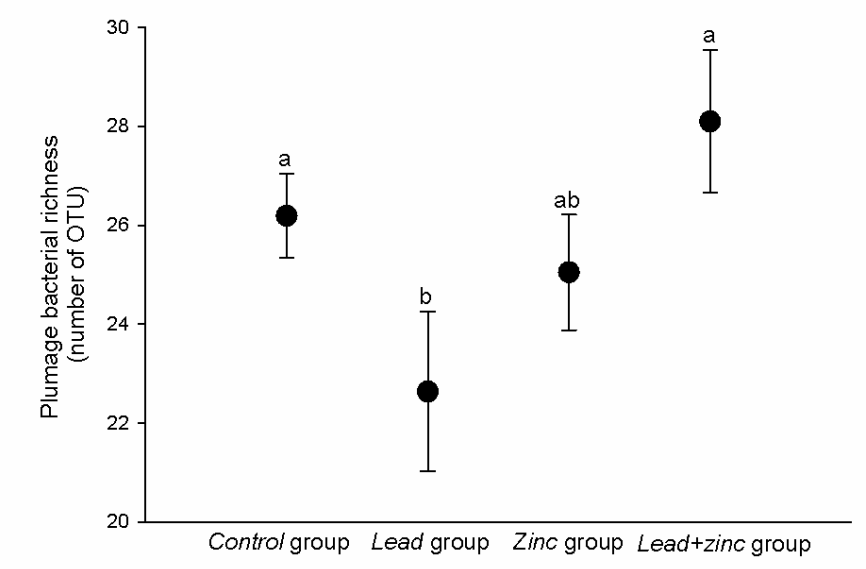


Fig. 3 Mean ± SE plumage bacterial richness (number of different otus) according to metal exposure. a and b were significantly different (p-value<0.05) while ab was not different from a or from b.

Plumage bacterial load was significantly lower among birds exposed to zinc (*zinc* and *lead+zinc* groups) than among the others (*lead* and *control* groups; W=280.5, P=0.029; Fig. 4). Although there was no significant difference between each pair of treatments (P>0.067), *zinc* group tended to have lower plumage bacterial loads than *control* group (W=29, P=0.072) and *lead* group (W=25, P=0.067). Lead did not significantly affect plumage bacterial load (W=181, P=0.623).

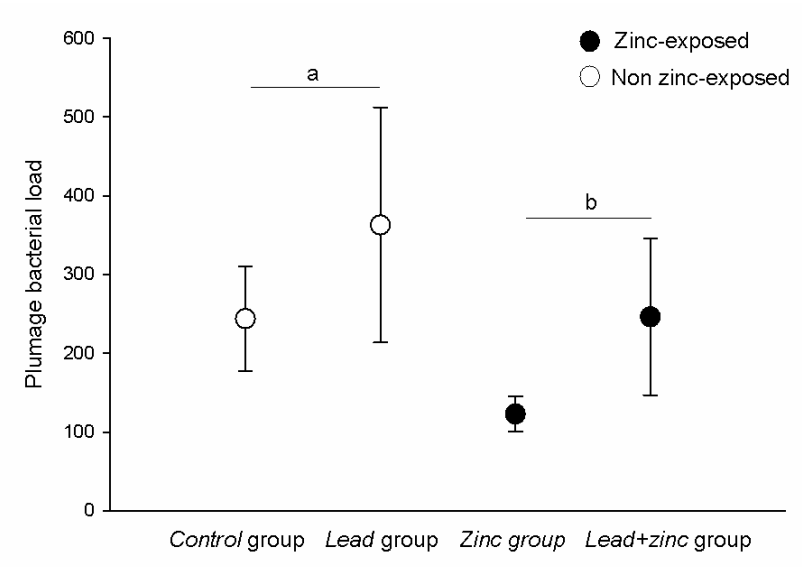


Fig. 4 Mean ± SE plumage bacterial load (number of bacterial colonies per slide) according to metal exposure. A (non-zinc-exposed) and b (zinc-exposed) were significantly different (p-value<0.05).

Finally, the time birds spent preening depended on the interaction between zinc and lead exposure ($Chi^2=3.97$, df=92, P=0.046). We performed partial models to compare each pairs of treatment. Although there was no significant difference between each pair of treatments (P>0.101), we graphically see that time spent preening appeared shorter among *lead* group than among the other ones (Fig. 5).

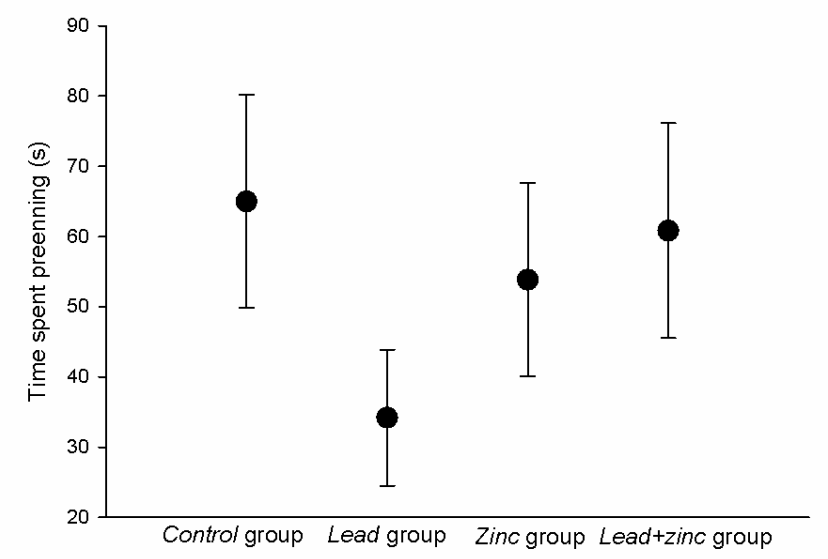


Fig. 5 Mean ± SE time (in seconds) spent preening during the five minute observation session according to metal exposure. Though a significant interaction between lead and zinc exposure was detected, there was no significant difference in each pairwise test.

Sex and plumage eumelanin level were retained in none of the tested models.

DISCUSSION

Trace metals may affect bird life-history traits by modulating their plumage bacterial community both through direct (e.g. Toxic effects on bacteria) and indirect effects (ie. By affecting bird behaviour and physiology). To test whether trace metals affect plumage bacterial community, we supplemented feral pigeons (*Columba livia*) with zinc and/or lead.

As expected, the composition of plumage bacterial community varied with metal exposure. The exposure to lead alone appears to induce the strongest effect. Indeed, plumage bacterial composition was more similar amongst the two aviaries with birds exposed to lead only (lead group; the first and fifth aviaries) than expected if considering a spatial effect only (n.b. Bacterial communities of birds in both aviaries were superimposed on Fig. 1). In addition, the plumage bacterial compositions of birds exposed to lead only were the most distant from the

communities of the other treatments. Moreover, although lead group did not have lower plumage bacterial load, they had reduced plumage bacterial richness compared to control group. These results suggest that lead may select for lead-tolerant plumage bacteria. To the best of our knowledge, our study is the first that provides evidence for the shaping effect of lead exposure on plumage bacterial community and is consistent with a previous study showing that lead alters the intestinal microbiome of mice (Breton et al. 2013). Moreover, lead group tended to preen less frequently than control group. Birds may decrease the time spent preening because the noxious effects of lead on their physiological state may not allow them to allocate in this costly behaviour (Dauwe et al. 2005, Eeva et al. 2009, Redig et al. 1991, Sens et al. 2003, Snoeijs et al. 2004, Trust et al. 1990, Wayland 2002). Moreover, pigeons may adjust the time spent preening according to their plumage bacterial community composition (Leclaire et al. 2014b, Moreno-Rueda 2010, Piault et al. 2008). Whatever the mechanism involved, by diminishing bird control on its plumage bacterial community, lead may change the dominant status of bacteria species and therefore induce the proliferation of species that were previously sensitive to preening secretions. High-throughput DNA sequence analysis would help identifying lead-tolerant bacteria species and therefore infer their potential pathogenicity and propensity to degrade feathers. More analysis should also be conducted to identify the proximal mechanisms involved in lead toxicity. For instance, in vitro exposure of feathers to these metals would allow us to split the direct and indirect effects that may induce the metals.

Like lead exposure, zinc exposure had toxic effects on the plumage bacterial community with birds exposed to zinc (zinc and lead+zinc groups) exhibiting lower bacterial load than other birds (lead and control groups). Similarly, high doses of zno decrease bacterial load and changes bacterial community in gastrointestinal tract of piglets (Hojberg et al. 2005, Vahjen et al. 2010), and inhibit bacterial growth in sludge and sediment (Cabrero et al. 1998, Vega-López et al. 2007). Zinc is known to be essential to several metabolic functions of bacteria (Sugarman 1983). At high concentration, zinc can, however, reduce protein and ATP content, interact with nucleic acids and enzyme active sites, decrease membrane health and eventually lead to cell necrosis (Martinez-Tabche and Gutierr 2000, Vega-López et al. 2007). Although the zinc concentration we used is within the natural range found in cities, it may be high enough to negatively affect feather bacteria and cause decreased bacterial load. Zinc may also affect feather bacteria load indirectly through its immunostimulating effect (Smith 2003). In feral pigeons, zinc have a positive effect on the production of specific antibodies (unpublished data), and may, therefore, increase the bactericidal capacity of uropygial secretions.

Interestingly, birds exposed to both lead and zinc exhibited the richest bacterial communities. As an edge effect, we could imagine that both zinc-tolerant and lead-tolerant species would develop in this environment.

Our results showed a strong effect of spatial proximity on bird plumage bacterial community, with birds in closer aviaries showing more similar bacterial communities. Despite our experiment was not aimed to test for this correlation, it shows the relatively small spatial scale transmission of plumage bacteria. While bacteria are likely transmitted through close contact (Kulkarni and Heeb 2007) and reciprocal delousing, some bacteria may be able to survive on non-feather substrate and, therefore, may disperse through indirect contact through the water used for bathing, perches, soil and the grids separating the aviaries to one another (Bisson et al. 2007). Because pigeons live in high density but have limited movement within their local environment, the plumage bacterial community of wild pigeons may, therefore, greatly vary between populations and lead to local coevolution and co-adaptation between the host and its bacterial community.

Our experimental exposure of feral pigeons to naturally-ranged concentrations of lead and/or zinc highlighted, for the first time, the effects of some trace metals commonly encountered in urban areas on plumage bacterial community. The birds used in our study were captured in Paris, and had been, therefore, previously exposed to trace metals in their natural urban habitat. Because ingested metals remains in organs and bones for a significant amount of time and in feathers until moulting (Agusa et al. 2005, Cosson et al. 1988, Gulson et al. 1996, Kim et al. 1998), plumage bacterial communities at the start of the experiment might have already been shaped by their past metal exposure. This would, however, have reduced the strength of our treatments, and the significant differences between treatments observed in our study are therefore conservative.

Although our knowledge on plumage bacterial community composition and function is scarce, it seems to play a role in bird fitness (Clayton 1999, Jacob et al. 2015, Shawkey et al. 2007). Indeed, some of these bacteria degrade the keratin of feathers, and thus negatively affect feather condition and colouration, which are involved in thermoregulation and visual communication (i.e. Dominant status, mate choice; (Hill and McGraw 2006, Wolf 2000)). For instance, feral pigeons and eastern bluebirds with higher bacterial load have paler iridescent neck feathers and higher feather brightness respectively (Leclaire et al. 2014b, Shawkey et al. 2007). Consequently, the effects of trace metals on feather bacterial community are likely to affect bird life-history traits. Further studies are now needed to determine how trace metals affect the host-bacterial community coevolution and modulate pigeon life-history traits.

ACKNOWLEDGMENTS

All experiments were carried out in strict accordance with the recommendations of the "European Convention for the Protection of vertebrate Animals used for Experimental and Other Scientific Purposes" and were conducted under the authorizations of the "Ministère de l'éducation nationale, de l'enseignement supérieur et de la recherche" (authorization N_00093.02) and the "Direction Départementale des Services Vétérinaires de Seine et-Marne" (authorization N_ 77-05). We thank the "Mairie de Paris" (Thomas Charachon) for allowing the capture of birds and the Centre de Recherche en Ecologie Expérimentale et Prédictive (CEREEP) which provided logistic support for the field work of this study. We are very thankful to T. Gayet, S. Pollet, S. Hasnaoui, F. Lorente, S. Perret and B. Decencière for their help all along the field work. Molecular analyses were partly performed at the technical facilities of the labex cemeb and were funded by an "ANR PDOC" grant (to SL) (No. ANR-13-PDOC-0002).

REFERENCES

Agusa, T., T. Matsumoto, T. Ikemoto, Y. Anan, R. Kubota, G. Yasunaga, T. Kunito, S. Tanabe, H. Ogi, and Y. Shibata. 2005. Body distribution of trace elements in black-tailed gulls from Rishiri Island, Japan: Age-dependent accumulation and transfer to feathers and eggs. Environmental Toxicology and Chemistry 24:2107–2120.

Archie, E. A., and K. R. Theis. 2011. Animal behaviour meets microbial ecology. Animal Behaviour 82:425–436.

Azimi, S., V. Rocher, M. Muller, R. Moilleron, and D. R. Thevenot. 2005. Sources, distribution and variability of hydrocarbons and metals in atmospheric deposition in an urban area (Paris, France). Science of The Total Environment 337:223–239.

Babich, H., and G. Stotzky. 1978. Toxicity of zinc to fungi, bacteria, and coliphages: influence of chloride ions. Applied and environmental microbiology 36:906–914.

Balcázar, J. L., D. Vendrell, I. De Blas, I. Ruiz-Zarzuela, O. Gironés, and J. L. Múzquiz. 2007. In vitro competitive adhesion and production of antagonistic compounds by lactic acid bacteria against fish pathogens. Veterinary Microbiology 122:373–380.

Bisson, I.-A., P. P. Marra, E. H. Burtt, M. Sikaroodi, and P. M. Gillevet. 2007. A molecular comparison of plumage and soil bacteria across biogeographic, ecological, and taxonomic scales. Microbial Ecology 54:65–81.

Breton, J., S. Massart, P. Vandamme, E. De Brandt, B. Pot, and B. Foligné. 2013. Ecotoxicology inside the gut: impact of heavy metals on the mouse microbiome. BMC Pharmacology and Toxicology 14:62.

Burkholder, K. M., K. L. Thompson, M. E. Einstein, T. J. Applegate, and J. A. Patterson. 2008. Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to *Salmonella enteritidis* colonization in broilers. Poultry Science 87:1734–1741.

Burtt Jr, E. H., and J. M. Ichida. 1999. Occurence of feather-degrading bacilli in the plumage of birds. The Auk 116:364–372.

Cabrero, A., S. Fernandez, F. Mirada, and J. Garcia. 1998. Effects of copper and zinc on the activated sludge bacteria growth kinetics. Water Research 32:1355–1362.

Chatelain, M., J. Gasparini, L. Jacquin, and A. Frantz. 2014. The adaptive function of melanin-based plumage coloration to trace metals. Biology Letters 10:20140164–20140164.

Clayton, D. H. 1999. Feather-busting bacteria. The Auk 116:302–304.

Cosson, R. P., J.-C. Amiard, and C. Amiard-Triquet. 1988. Trace elements in little egrets and flamingos of Camargue, France. Ecotoxicology and Environmental Safety 15:107–116.

Czirják, G. Á., P. L. Pap, C. I. Vágási, M. Giraudeau, C. Mureșan, P. Mirleau, and P. Heeb. 2013. Preen gland removal increases plumage bacterial load but not that of feather-degrading bacteria. Naturwissenschaften 100:145–151.

Dauwe, L. Bervoets, R. Blust, M. Ee, T. 2002. Tissue levels of lead in experimentally exposed zebra finches (*Taeniopygia guttata*) with particular attention on the use of feathers as biomonitors. Archives of Environmental Contamination and Toxicology 42:88–92.

Dauwe, T., E. Janssens, R. Pinxten, and M. Eens. 2005. The reproductive success and quality of blue tits (*Parus caeruleus*) in a heavy metal pollution gradient. Environmental Pollution 136:243–251.

Dotterud, L. K., T. Wilsgaard, L. H. Vorland, and E. S. Falk. 2008. The effect of UVB radiation on skin microbiota in patients with atopic dermatitis and healthy controls. International Journal of Circumpolar Health 67.

Eeva, T., M. Ahola, and E. Lehikoinen. 2009. Breeding performance of blue tits (Cyanistes caeruleus) and great tits (Parus major) in a heavy metal polluted area. Environmental Pollution 157:3126–3131.

Frank, D. N., C. E. Robertson, C. M. Hamm, Z. Kpadeh, T. Zhang, H. Chen, W. Zhu, R. B. Sartor, E. C. Boedeker, N. Harpaz, et al. 2011. Disease phenotype and genotype are associated with

shifts in intestinal-associated microbiota in inflammatory bowel diseases: Inflammatory Bowel Diseases 17:179–184.

Frantz, A., M.-A. Pottier, B. Karimi, H. Corbel, E. Aubry, C. Haussy, J. Gasparini, and M. Castrec-Rouelle. 2012. Contrasting levels of heavy metals in the feathers of urban pigeons from close habitats suggest limited movements at a restricted scale. Environmental Pollution 168:23–28.

Giller, K. E., E. Witter, and S. P. Mcgrath. 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biology and Biochemistry 30:1389–1414.

Griffiths, R., M. C. Double, K. Orr, and R. J. G. Dawson. 1998. A DNA test to sex most birds. Molecular Ecology 7:1071–1075.

Gulson, B., K. J. Mizon, M. J. Korsch, D. Howarth, A. Phillips, and J. Hall. 1996. Impact on blood lead in children and adults following relocation from their source of exposure and contribution of skeletal tissue to blood lead. Bulletin of Environmental Contamination and Toxicology 56:543–550.

Gunderson, A. R., M. H. Forsyth, and J. P. Swaddle. 2009. Evidence that plumage bacteria influence feather coloration and body condition of eastern bluebirds *Sialia sialis*. Journal of Avian Biology 40:440–447.

Hildebrand, F., T. L. Nguyen, B. Brinkman, R. G. Yunta, B. Cauwe, P. Vandenabeele, A. Liston, and J. Raes. 2013. Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. Genome Biol 14:R4.

Hill, G. E., and K. J. Mcgraw. 2006. Bird coloration. Harvard University Press, Cambridge, Mass.

Hill, M. 1997. Intestinal flora and endogenous vitamin synthesis. European Journal of Cancer Prevention 6.

Hojberg, O., N. Canibe, H. D. Poulsen, M. S. Hedemann, and B. B. Jensen. 2005. Influence of dietary zinc oxide and copper sulfate on the gastrointestinal ecosystem in newly weaned piglets. Applied and Environmental Microbiology 71:2267–2277.

Jacob, S., N. Parthuisot, A. Vallat, F. Ramon-Portugal, F. Helfenstein, and P. Heeb. 2015. Microbiome affects egg carotenoid investment, nestling development and adult oxidative costs of reproduction in great tits. Functional Ecology:n/a–n/a.

Jacquin, L., P. Lenouvel, C. Haussy, S. Ducatez, and J. Gasparini. 2011. Melanin-based coloration is related to parasite intensity and cellular immune response in an urban free living bird: the feral pigeon *Columba livia*. Journal of Avian Biology 42:11–15.

Johnston, R., and M. Janiga. 1995. Feral pigeons. Oxford University Press.

Kekkonen, J., I. K. Hanski, R. A. Väisänen, and J. E. Brommer. 2012. Levels of heavy metals in house sparrows (*Passer domesticus*) from urban and rural habitats of southern Finland. Ornis Fennica 89.

Kim, E. Y., R. Goto, S. Tanabe, H. Tanaka, and R. Tatsukawa. 1998. Distribution of 14 elements in tissues and organs of oceanic seabirds. Archives of Environmental Contamination and Toxicology 35:638–645.

Kulkarni, S., and P. Heeb. 2007. Social and sexual behaviours aid transmission of bacteria in birds. Behavioural Processes 74:88–92.

Leclaire, S., G. Czirjak, A. Hammouda, and J. Gasparini. 2015. Feather bacterial load shapes the trade-off between preening and immunity in pigeons. BMC Evolutionary Biology 15:60.

Leclaire, S., J. F. Nielsen, and C. M. Drea. 2014a. Bacterial communities in meerkat anal scent secretions vary with host sex, age, and group membership. Behavioral Ecology 25:996–1004.

Leclaire, S., P. Pierret, M. Chatelain, and J. Gasparini. 2014b. Feather bacterial load affects plumage condition, iridescent color, and investment in preening in pigeons. Behavioral Ecology 25:1192–1198.

Liu, Y., Y. Li, K. Liu, and J. Shen. 2014. Exposing to Cadmium Stress Cause Profound Toxic Effect on Microbiota of the Mice Intestinal Tract. Plos ONE 9:e85323.

Martinez-Tabche, L., and I. Gutierr. 2000. Toxic effects of zinc from trout farm sediments on ATP, protein, and hemoglobin concentrations of *Limnodrilus hoffmeisteri*. Journal of Toxicology and Environmental Health, Part A 59:575–583.

Moreno-Rueda, G. 2010. Uropygial gland size correlates with feather holes, body condition and wingbar size in the house sparrow *Passer domesticus*. Journal of Avian Biology 41:229–236.

Mueller, S., K. Saunier, C. Hanisch, E. Norin, L. Alm, T. Midtvedt, A. Cresci, S. Silvi, C. Orpianesi, M. C. Verdenelli, et al. 2006. Differences in fecal microbiota in different european study populations in relation to age, gender, and country: a cross-sectional study. Applied and Environmental Microbiology 72:1027–1033.

Muza, M. M., E. H. Burtt, and J. M. Ichida. 2000. Distribution of bacteria on feathers of some Eastern North American birds. The Wilson Bulletin 112:432–435.

Oh, S., S.-H. Kim, Y. Ko, J.-H. Sim, K. S. Kim, S.-H. Lee, S. Park, and Y. J. Kim. 2006. Effect of bacteriocin produced by *Lactococcus sp.* HY 449 on skin-inflammatory bacteria. Food and Chemical Toxicology 44:552–559.

Oksanen, J., R. Kindt, P. Legendre, B. O'Hara, M. Stevens, M. Oksanen, and M. Suggests. 2007. Vegan: Community Ecology Package. Available from: http//CRAN.R-project.org/package=vegan.

Olsson, J. C., A. Westerdahl, P. L. Conway, and S. Kjelleberg. 1992. Intestinal colonization potential of turbot (*Scophthalmus maximus*)-and dab (*Limanda limanda*)-associated bacteria with inhibitory effects against *Vibrio anguillarum*. Applied and Environmental Microbiology 58:551–556.

Pattee, O. H. 1984. Eggshell thickness and reproduction in American kestrels exposed to chronic dietary lead. Archives of environmental contamination and toxicology 13:29–34.

Piault, R., J. Gasparini, P. Bize, M. Paulet, K. J. Mcgraw, and A. Roulin. 2008. Experimental support for the makeup hypothesis in nestling tawny owls (*Strix aluco*). Behavioral Ecology 19:703–709.

Pilastro, A., L. Congiu, L. Tallandini, and M. Turchetto. 1993. The use of bird feathers for the monitoring of cadmium pollution. Archives of Environmental Contamination and Toxicology 24:355–358.

Prasad, A. S. 1998. Zinc and immunity. Molecular and cellular biochemistry 188:63–69.

Ranjard, L., E. Brothier, and S. Nazaret. 2000. Sequencing bands of ribosomal intergenic spacer analysis fingerprints for characterization and microscale distribution of soil bacterium populations responding to mercury spiking. Applied and Environmental Microbiology 66:5334–5339.

Redig, P. T., E. M. Lawler, S. Schwartz, J. L. Dunnette, B. Stephenson, and G. E. Duke. 1991. Effects of chronic exposure to sublethal concentrations of lead acetate on heme synthesis and immune function in red-tailed hawks. Archives of Environmental Contamination and Toxicology 21:72–77.

Reid, C., K. Mcinnes, J. M. Mclelland, and B. D. Gartrell. 2012. Anthropogenic lead (Pb) exposure in populations of a wild parrot (kea *Nestor notabilis*). New Zealand Journal of Ecology 36:56.

Rosenthal, M., D. Goldberg, A. Aiello, E. Larson, and B. Foxman. 2011. Skin microbiota: Microbial community structure and its potential association with health and disease. Infection, Genetics and Evolution 11:839–848.

Roux, K. E., and P. P. Marra. 2007. The presence and impact of environmental lead in passerine birds along an urban to rural land use gradient. Archives of Environmental Contamination and Toxicology 53:261–268.

Ruiz-de-Castañeda, R., A. I. Vela, E. Lobato, V. Briones, and J. Moreno. 2011. Bacterial loads on eggshells of the pied flycatcher: environmental and maternal factors. The Condor 113:200–208.

Ruiz-Rodriguez, M., E. Valdivia, J. J. Soler, M. Martin-Vivaldi, A. M. Martin-Platero, and M. Martinez-Bueno. 2009. Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation. Journal of Experimental Biology 212:3621–3626.

Scheifler, R., M. Cœurdassier, C. Morilhat, N. Bernard, B. Faivre, P. Flicoteaux, P. Giraudoux, M. Noël, P. Piotte, D. Rieffel, et al. 2006. Lead concentrations in feathers and blood of common blackbirds (*Turdus merula*) and in earthworms inhabiting unpolluted and moderately polluted urban areas. Science of The Total Environment 371:197–205.

Sens, E. J., T. Dauwe, R. Pinxten, and M. Eens. 2003. Breeding performance of great tits (*Parus major*) along a gradient of heavy metal pollution. Environmental toxicology and chemistry 22:1140–1145.

Shawkey, M. D., and G. E. Hill. 2004. Feathers at a fine scale. The Auk 121:652.

Shawkey, M. D., S. R. Pillai, and G. E. Hill. 2003. Chemical warfare? Effects of uropygial oil on feather-degrading bacteria. Journal of Avian Biology 34:345–349.

Shawkey, M. D., S. R. Pillai, G. E. Hill, L. M. Siefferman, and S. R. Roberts. 2007. Bacteria as an agent for change in structural plumage color: correlational and experimental evidence. The American Naturalist 169:S112–S121.

Smith, M. O. 2003. Effects of different levels of zinc on the performance and immunocompetence of broilers under heat stress. Poultry Science 82:1580–1588.

Snoeijs, T., T. Dauwe, R. Pinxten, F. Vandesande, and M. Eens. 2004. Heavy metal exposure affects the humoral immune response in a free-living small songbird, the great tit (*Parus major*). Archives of Environmental Contamination and Toxicology 46.

Sugarman, B. 1983. Zinc and Infection. Clinical Infectious Diseases 5:137–147.

Trust, K. A., M. W. Miller, J. K. Ringelman, and I. M. Orme. 1990. Effects of ingested lead on antibody production in mallards (*Anas platyrhynchos*). Journal of Wildlife Diseases 26:316–322.

Vahjen, W., R. Pieper, and J. Zentek. 2010. Bar-coded pyrosequencing of 16S rRNA gene amplicons reveals changes in ileal porcine bacterial communities due to high dietary zinc intake. Applied and Environmental Microbiology 76:6689–6691.

Vega-López, A., E. Amora-Lazcano, E. López-López, O. Terrón, and J. B. Proal-Nájera. 2007. Toxic effects of zinc on anaerobic microbiota from Zimapán Reservoir (Mexico). Anaerobe 13:65–73.

Wayland, M. 2002. Immune function, stress response, and body condition in arctic-breeding common eiders in relation to cadmium, mercury, and selenium concentrations. Environmental Research 90:47–60.

Whitaker, J. M., D. A. Cristol, and M. H. Forsyth. 2005. Prevalence and genetic diversity of *Bacillus licheniformis* in avian plumage. Journal of Field Ornithology 76:264–270.

Wolf, B. O. 2000. The role of the plumage in heat transfer processes of birds. Integrative and Comparative Biology 40:575–584.

Yorinks, N., and C. T. Atkinson. 2000. Effects of malaria on activity budgets of experimentally infected juvenile apapane (*Himatione sanguinea*). The Auk 117:731.

Conclusion générale

Le but de mes recherches était premièrement de tester les effets écotoxicologiques d'une exposition aux métaux traces, deuxièmement de tester le rôle de la mélanine dans la fixation des métaux traces, leur détoxication, ainsi que dans la sensibilité des individus aux métaux traces, et troisièmement de comparer les réponses écophysiologiques des individus en fonction de la coloration mélanique de leur plumage, ceci indépendamment de leur exposition aux métaux traces.

Quels sont les effets écotoxicologiques d'une exposition chronique aux métaux traces ?

D'un point de vue écotoxicologique, l'ensemble de nos résultats corroborent la toxicité du plomb d'une part, et les bénéfices du zinc d'autre part, à la fois sur le maintien de la corpulence, la reproduction, l'immunité et les transferts maternels. Lors des expériences menées, les concentrations auxquelles ont été soumis les individus étaient clairement dans le bas de la gamme des concentrations environnementales rencontrées en milieu urbain. Aussi, nous nous attendons à des effets encore plus marqués *in natura*, d'autant plus qu'en milieu naturel, les individus sont soumis à des facteurs de stress multiples (e.g. prédation, parasitisme, compétition intraspécifique pour l'accès aux partenaires sexuels, les sites de nidification ou encore la nourriture), susceptibles d'augmenter les compromis existants entre l'ensemble des fonctions biologiques (e.g. immunité, croissance, reproduction) et d'accentuer ainsi les coûts induits par les métaux traces. La mesure de l'exposition réelle des individus aux métaux traces dans leur environnement naturel est extrêmement difficile puisque les métaux peuvent être absorbés par différentes voies ; estimer cette exposition est une étape importante dans l'étude des effets écotoxicologiques des métaux traces.

Bien que les régimes alimentaires et donc l'exposition aux métaux traces via l'alimentation diffèrent selon les espèces, les conclusions de notre étude devraient pouvoir être applicables à l'ensemble des espèces aviaires urbaines, voire des mammifères, reptiles et amphibiens terrestres. Aussi, les métaux traces sont susceptibles de représenter une pression de sélection bien réelle pour les populations urbaines, leur fonctionnement et leur dynamique. Notamment, les effets écotoxicologiques mesurées sont susceptibles d'être plus marqués chez les espèces se trouvant en haut des réseaux trophiques, comme les espèces carnivores ou piscivores, du fait du phénomère de bioamplification (voir Introduction). Alors que le pigeon biset n'est pas une espèce dont les effectifs diminuent, plusieurs espèces communes, comme le moineau domestique (Shaw

et al., 2008), voient leur abondance diminuer à l'échelle de l'Europe ; d'autres encore, comme le faucon crécerelle, sont relativement rares et font l'objet de programmes de conservation. Bien que ses émissions atmosphériques aient grandement diminué, le plomb reste un polluant majeur en milieu urbain et les efforts mis en place pour la remédiation des sols prennent toute leur importance.

Les métaux traces sont relativement nombreux en milieu urbain (e.g. cadmium, cuivre, aluminium, etc.). Notre étude a testé les effets de seulement deux d'entre eux. D'autres études doivent donc être menées pour identifier les coûts et les bénéfices de chacun. Par ailleurs, les populations urbaines sont exposées à l'ensemble de ces métaux de façon simultanée. Les effets antagonistes du plomb et du zinc mis en évidence dans notre étude soulèvent l'importance de comprendre comment les métaux traces interagissent entre eux puisque c'est bien la somme de leurs effets qui détermineront leur impact global sur les populations.

Certaines populations, comme celles de pigeons bisets, se maintiennent très bien en milieu urbain, ceci malgré leur exposition à des métaux traces toxiques. Aussi, outre le transfert des métaux traces dans le plumage, on peut imaginer que d'autres mécanismes physiologiques (e.g. synthèse d'enzymes de détoxication et de transferrines, production d'antioxydants comme le glutathion et la superoxyde dismutase) d'acclimatation ou d'adaptation, permettant une meilleure tolérance aux métaux traces, aient évolué. Il serait donc intéressant de comparer ces paramètres physiologiques au sein de plusieurs populations urbaines et rurales, à la fois dans un environnement pollué en métaux traces et dans un environnement sain.

Le mélanisme du plumage est-il lié à la capacité à séquestrer les métaux dans les plumes ?

La sensibilité des individus aux métaux traces est variable. Aussi, nous pouvons faire l'hypothèse que les individus davantage tolérants aient une meilleure aptitude phénotypique. Notre étude montre que les individus au plumage le plus mélanique sont capables de transférer de plus grandes quantités de zinc et de plomb dans leur plumage. Plusieurs métaux traces pouvant occuper un même site de fixation sur la mélanine (Hong and Simon, 2007; Liu et al., 2004), il serait intéressant de savoir si, lorsqu'un individu est soumis à un taux d'exposition constant à un certain métal, la proportion qui est séquestrée dans le plumage dépend de l'exposition de l'individu à d'autres métaux traces. En effet, selon le degré d'affinité des métaux pour ces sites de fixation, la proportion séquestrée n'est peut-être pas corrélée au taux d'exposition.

Le mélanisme du plumage permet-il la détoxication des métaux traces ?

Alors que le transfert de métaux potentiellement toxiques dans des parties inertes comme les plumes pourrait représenter un moyen efficace de détoxication, notre étude n'a pas réussi à le mettre en évidence ; les individus au plumage le plus mélanique, ne montraient ni des concentrations internes en métaux traces plus basses que celles des individus les plus clairs, ni des paramètres écophysiologiques témoignant de leur meilleure tolérance. Néanmoins, les concentrations sanguines en métaux traces, mesurées dans notre étude, ne seraient *a priori* pas de bon estimateurs de l'exposition récente des individus. D'autres mesures doivent être développées, notamment prenant en compte les concentrations dans les organes internes, afin d'estimer de façon appropriée la détoxication. En ce qui concerne notre étude, nous ne pouvons pas être certains que l'absence de différence au niveau des concentrations sanguines en métaux traces selon le degré d'eumélanisme résulte de l'inadaptation de la mesure utilisée ou d'une absence réelle de l'efficacité de la détoxication. Il est possible que l'apport en métaux traces par l'environnement soit nettement supérieur à la capacité de détoxication du plumage. Le transfert des métaux traces dans les plumes devrait être plus important durant l'automne (correspondant au pic de la mue chez le pigeon biset et la plupart des oiseaux) et chez les juvéniles, dont la croissance des plumes est synchronisée. Aussi, si détoxication il y a, il serait plus probable de la mettre en évidence durant ces deux périodes.

Le mélanisme du plumage module-t-il les effets écotoxicologiques induits par les métaux traces ?

En dépit du fait que les concentrations sanguines en métaux traces ne diffèrent pas entre les individus selon qu'ils sont plus ou moins mélaniques, plusieurs interactions entre le degré d'eumélanisme du plumage et l'exposition au plomb mesurées dans notre étude suggèrent à première vue, et contrairement à notre hypothèse, un désavantage au mélanisme du plumage dans un environnement pollué en métaux traces. Dans les deux cas, des hypothèses alternatives peuvent être soulevées et soulignent la complexité des réponses physiologiques impliquées. Notamment, il apparait important de comprendre les effets du plomb sur le système endocrinien, et notamment la synthèse de corticoïdes. De plus, la toxicité du plomb résultant en partie du stress oxydatif qu'il engendre, l'étude de ce paramètre physiologique pourrait permettre la mise en évidence des effets bénéfiques suggérés du mélanisme. Par ailleurs, il apparait essentiel de mieux comprendre les mécanismes de transfert dans le plumage des métaux traces essentiels comme le zinc. En effet, dans le cas où seul l'excédent serait séquestré, alors le transfert serait bénéfique ; au contraire, si les concentrations internes de ces éléments ne sont pas strictement régulées, alors leur transfert pourrait résulter en des carences et représenterait un coût pour l'individu. Dans ce second cas, la valeur adaptative du mélanisme, dont nous faisons l'hypothèse,

dépendrait des concentrations environnementales relatives des différents métaux ; ce compromis pourrait alors expliquer la plus forte fréquence de pigeons fortement mélaniques en milieu urbain par rapport au milieu rural.

Malgré le manque de résultats nous permettant de soutenir le rôle adaptatif du mélanisme dans un environnement pollué en métaux traces, la plus forte fréquence de juvéniles au plumage fortement mélanique chez les individus exposés au plomb suggère bien un avantage au mélanisme dans un tel environnement. Le mécanisme sous-jacent à cette survie préférentielle, qu'ils impliquent une détoxication du plomb ou des effets pléiotropes reste à déterminer.

Outre la mélanine transférée dans les phanères, de la mélanine est également accumulée au niveau de la peau ou dans les organes, c'est-à-dire dans des parties vivantes. Certains auteurs suggèrent qu'il puisse y avoir une coévolution de la mélanine interne et externe (Dubey and Roulin, 2014) ; par exemple, chez un oiseau, plus son plumage est mélanique, plus ses organes (e.g. foie, reins,) le sont également. Alors que l'accumulation de métaux traces au niveau de la mélanine du plumage n'apparaît que bénéfique, leur accumulation au niveau des organes pourrait être délétère. Aussi, et contrairement à notre hypothèse, le mélanisme du plumage pourrait être désavantageux dans un environnement pollué en métaux traces, du fait d'une forte accumulation de métaux dans les organes internes. Bien que cette hypothèse alternative doive être prise en compte pour permettre la compréhension des résultats de notre étude, les reins et foies des pigeons utilisés dans nos expériences présentes des concentrations non significatives d'eumélanine et de phéomélanine (résultats non présentés).

Le mélanisme du plumage est-il représentatif de différences physiologiques entre les individus ?

Notre étude met en évidence plusieurs différences physiologiques existantes entre les individus eumélaniques et les individus phéomélaniques, plus particulièrement au niveau de l'immunité et du transfert du zinc dans le plumage. Ces deux résultats suggèrent une allocation plus importante d'énergie dans la réponse à une infection et des besoins plus importants en zinc chez les individus phéomélaniques. Bien que la mise en place d'une réponse immunitaire puisse être coûteuse et désavantageuse, notre étude ne souligne pas de coûts et bénéfices clairs associés au phéomélanisme et alimente peu le débat sur l'évolution de ce pigment (Hill and Hill, 2000). Rares sont les études comparant les coûts et les bénéfices associés à l'eumélanisme et au phéomélanisme (Galván and Solano, 2009; Roulin et al., 2011; Samokhvalov et al., 2007; Zduniak et al., 2014). Ces études sont en effet difficiles à mener, entre autres du fait que peu d'espèces présentent les deux formes de mélanisme. Aussi, et bien que la fréquence des individus phéomélaniques est faible par rapport à celle des individus eumélaniques, le pigeon

biset apparait comme un modèle de choix pour tester ces problématiques. D'autres études doivent être menées chez cette espèce. Elles pourraient ainsi permettre de révéler des différences physiologiques significatives, que nous n'avons pas pu mettre en évidence dans nos études, du fait d'un plan d'expérimentation déséquilibré et ainsi une sous-représentation des individus phéomélaniques.

Outre des différences entre individus eumélaniques et phéomélaniques, nous nous attendions à d'éventuelles corrélations entre le degré d'eumélanisme du plumage et certains paramètres physiologiques (e.g. la réponse immunitaire), ceci quel que soit l'exposition des individus aux métaux traces. En effet, des liens génétiques existeraient entre l'activation de la synthèse de la mélanine et celle d'autres paramètres biologiques (e.g. immunité, tolérance au stress oxydatif et physiologique; Ducrest et al., 2008). Une telle covariation a seulement été mesurée pour le transfert des lysozymes, les femelles les plus mélaniques transférant d'avantage de lysozymes dans leurs œufs que les femelles les plus pâles. Aussi, il apparait important de relativiser le poids des effets pléiotropes dans la mesure des coûts et bénéfices associés au degré d'eumélanisme, susceptible de dépendre de l'environnement, et peut-être de la condition des individus (i.e. les effets pléiotropes pourraient ne pas être observables dans le cas où les compromis entre les différentes fonctions biologiques sont faibles).

L'évolution du mélanisme dans le règne animal

Bien que le pigeon biset présente un polymorphisme au niveau de la coloration mélanique de son plumage particulièrement important, de nombreuses autres espèces montrent des variations dans le degré de mélanisme de leur plumage (e.g. mésange charbonnière) mais aussi de leur pelage (e.g. écureuil gris) ou leurs écailles (e.g. vipère aspic, phalène du bouleau). Malheureusement, selon le taxon, peu d'études, voir aucune à ma connaissance ne s'est intéressée à la variation de la coloration mélanique des phanères le long d'un gradient (voir néanmoins Cook, 2000; Dauwe and Eens, 2008; L. Jacquin et al., 2013) d'urbanisation. Les mécanismes de fixation des métaux traces à la mélanine étant très certainement identiques chez l'ensemble de ces espèces, nos résultats pourraient s'y appliquer. Notons néanmoins que l'efficacité de la détoxication supposée dans notre étude dépendrait avant tout du ratio entre l'absorption des métaux traces et leur élimination, c'est-à-dire de l'exposition aux métaux traces présents dans l'environnement, du degré de mélanisme des phanères et de la fréquence de leur renouvellement, variable selon les organismes.

Afin d'étudier l'évolution de la coloration mélanique des phanères, il est essentiel de prendre en considération l'ensemble des pressions de sélection susceptibles d'influencer cette évolution.

Alors que les métaux traces pourraient, d'après notre étude, constituer une de ces pressions, de nombreuses autres semblent également impliquées. La plus part des études suggèrent que les avantages et inconvénients du mélanisme sont indirects et résultent des liens génétiques existants entre la synthèse de la mélanine et de nombreux autres paramètres biologiques (Ducrest et al., 2008). Ainsi, les oiseaux davantage mélaniques seraient plus résistants au parasitisme (Jacquin et al., 2011; Jacquin et al., 2013), au stress oxydatif (Roulin et al., 2011) et au stress physiologique (Almasi et al., 2010, 2008). Le mélanisme est également un paramètre influençant fortement les capacités de thermorégulation, avant tout chez les ectothermes (Clusella Trullas et al., 2007). Par ailleurs, la prédation a un rôle important dans la sélection de phénotypes moins visibles et ainsi l'évolution du camouflage et du mimétisme (Cook, 2000). Chez le pigeon biset, la sélection de la coloration du plumage serait également fréquence-dépendante, leurs prédateurs attaquant préférentiellement les morphes les plus distinguables (Rutz, 2012). Finalement, les plumes les plus mélaniques sont davantage résistantes aux bactéries kératinophages (Goldstein et al., 2004) ; certains auteurs suggèrent même que cette propriété expliquerait la loi de Gloger, selon laquelle le plumage est davantage foncé en milieux humides, environnement très propice à la prolifération bactérienne (Burtt and Ichida, 1999). Outre les forces de sélection, l'évolution de la coloration mélanique des phanères peut dépendre de phénomènes de dérives liés à des effets fondateurs et des taux de migration faibles (Bittner and King, 2003). Ces facteurs sont susceptibles d'avoir des effets synergiques ou antagonistes et c'est bien de la somme de ces effets que découlent les différences de fréquences au niveau de la coloration mélanique des phanères.

Références bibliographiques

Adout, A., Hawlena, D., Maman, R., Paz-Tal, O., Karpas, Z., 2007. Determination of trace elements in pigeon and raven feathers by ICPMS. Int. J. Mass Spectrom. 267, 109–116. doi:10.1016/j.ijms.2007.02.022

Agusa, T., Matsumoto, T., Ikemoto, T., Anan, Y., Kubota, R., Yasunaga, G., Kunito, T., Tanabe, S., Ogi, H., Shibata, Y., 2005. Body distribution of trace elements in black-tailed gulls from Rishiri Island, Japan: Age-dependent accumulation and transfer to feathers and eggs. Environ. Toxicol. Chem. 24, 2107–2120.

Almasi, B., Jenni, L., Jenni-Eiermann, S., Roulin, A., 2010. Regulation of stress response is heritable and functionally linked to melanin-based coloration. J. Evol. Biol. 23, 987–996. doi:10.1111/j.1420-9101.2010.01969.x

Almasi, B., Roulin, A., Jenni-Eiermann, S., Jenni, L., 2008. Parental investment and its sensitivity to corticosterone is linked to melanin-based coloration in barn owls. Horm. Behav. 54, 217–223. doi:10.1016/j.yhbeh.2008.02.021

Almasi, B., Roulin, A., Korner-Nievergelt, F., Jenni-Eiermann, S., Jenni, L., 2012. Coloration signals the ability to cope with elevated stress hormones: effects of corticosterone on growth of barn owls are associated with melanism: Plumage coloration and stress sensitivity. J. Evol. Biol. 25, 1189–1199. doi:10.1111/j.1420-9101.2012.02508.x

Alscher, R.G., 1989. Biosynthesis and antioxidant function of glutathione in plants. Physiol. Plant. 77, 457–464. doi:10.1111/j.1399-3054.1989.tb05667.x

Azimi, S., Ludwig, A., Thévenot, D.R., Colin, J.-L., 2003. Trace metal determination in total atmospheric deposition in rural and urban areas. Sci. Total Environ. 308, 247–256.

Azimi, S., Rocher, V., Muller, M., Moilleron, R., Thevenot, D.R., 2005. Sources, distribution and variability of hydrocarbons and metals in atmospheric deposition in an urban area (Paris, France). Sci. Total Environ. 337, 223–239. doi:10.1016/j.scitotenv.2004.06.020

Babich, H., Stotzky, G., 1978. Toxicity of zinc to fungi, bacteria, and coliphages: influence of chloride ions. Appl. Environ. Microbiol. 36, 906–914.

Baos, R., Blas, J., Bortolotti, G.R., Marchant, T.A., Hiraldo, F., 2006. Adrenocortical response to stress and thyroid hormone status in free-living nestling white storks (*Ciconia ciconia*) exposed to heavy metal and arsenic contamination. Environ. Health Perspect. 114, 1497–1501. doi:10.1289/ehp.9099

Benathan, M., Alvero-Jackson, H., Mooy, A., Scaletta, C., Brothier, E., 1992. Relationship between melanogenesis, glutathione levels and melphalan toxicity in human melanoma cells. Melanoma Res. 2, 283–411.

Benathan, M., Virador, V., Furumura, M., Kobayashi, N., Panizzon, R.G., Hearing, V.J., 1999. Co-regulation of melanin precursors and tyrosinase in human pigment cells: roles of cysteine and glutathione. Cell. Mol. Biol. Noisy--Gd. Fr. 45, 981–990.

Berglund, A., Sturve, J., Forlin, L., Nyholm, N., 2007. Oxidative stress in pied flycatcher (*Ficedula hypoleuca*) nestlings from metal contaminated environments in northern Sweden. Environ. Res. 105, 330–339. doi:10.1016/j.envres.2007.06.002

Bertine, K.K., Goldberg, E.D., 1971. Fossil fuel combustion and the major sedimentary cycle. Science 173, 233–235. doi:10.1126/science.173.3993.233

Biasioli, M., Barberis, R., Ajmonemarsan, F., 2006. The influence of a large city on some soil properties and metals content. Sci. Total Environ. 356, 154–164. doi:10.1016/j.scitotenv.2005.04.033

Bilos, C., Colombo, J.., Skorupka, C.., Rodriguez Presa, M.., 2001. Sources, distribution and variability of airborne trace metals in La Plata City area, Argentina. Environ. Pollut. 111, 149–158. doi:10.1016/S0269-7491(99)00328-0

Bittner, T.D., King, R.B., 2003. Gene flow and melanism in garter snakes revisited: a comparison of molecular markers and island vs. coalescent models. Biol. J. Linn. Soc. 79, 389–399. doi:10.1046/j.1095-8312.2003.00199.x

Bókony, V., Garamszegi, L.Z., Hirschenhauser, K., Liker, A., 2008. Testosterone and melanin-based black plumage coloration: a comparative study. Behav. Ecol. Sociobiol. 62, 1229–1238. doi:10.1007/s00265-008-0551-2

Bonneaud, C., Mazuc, J., Gonzalez, G., Haussy, C., Chastel, O., Faivre, B., Sorci, G., 2003. Assessing the cost of mounting an immune response. Am. Nat. 161, 367–379. doi:10.1086/346134

Boulinier, T., Staszewski, V., 2008. Maternal transfer of antibodies: raising immuno-ecology issues. Trends Ecol. Evol. 23, 282–288. doi:10.1016/j.tree.2007.12.006

Bressler, J., Kim, K., Chakraborti, T., Goldstein, G., 1999. Molecular mechanisms of lead neurotoxicity. Neurochem. Res. 24, 595–600.

Breton, J., Massart, S., Vandamme, P., De Brandt, E., Pot, B., Foligné, B., 2013. Ecotoxicology inside the gut: impact of heavy metals on the mouse microbiome. BMC Pharmacol. Toxicol. 14, 62. doi:10.1186/2050-6511-14-62

Bridelli, M.G., Crippa, P.R., 2007. Theoretical analysis of the adsorption of metal ions to the surface of melanin particles. Adsorption 14, 101–109. doi:10.1007/s10450-007-9059-8

Burger, J., Gochfeld, M., 2004. Effects of lead and exercise on endurance and learning in young herring gulls. Ecotoxicol. Environ. Saf. 57, 136–144. doi:10.1016/S0147-6513(03)00035-6

Burkholder, K.M., Thompson, K.L., Einstein, M.E., Applegate, T.J., Patterson, J.A., 2008. Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to *Salmonella enteritidis* colonization in broilers. Poult. Sci. 87, 1734–1741. doi:10.3382/ps.2008-00107

Burtt Jr, E.H., Ichida, J.M., 1999. Occurence of feather-degrading bacilli in the plumage of birds. The Auk 116, 364–372.

Cerklewski, F.L., Forbes, R.M., 1976. Influence of dietary zinc on lead toxicity in the rat. J. Nutr. 106, 689–696.

Chandra, R.K., Dayton, D.H., 1982. Trace element regulation of immunity and infection. Nutr. Res. 2, 721–733. doi:10.1016/S0271-5317(82)80116-4

Chatelain, M., Gasparini, J., Jacquin, L., Frantz, A., 2014. The adaptive function of melanin-based plumage coloration to trace metals. Biol. Lett. 10, 20140164–20140164. doi:10.1098/rsbl.2014.0164

Chen, T.B., Wong, J.W.C., Zhou, H.Y., Wong, M.H., 1997. Assessment of trace metal distribution and contamination in surface soils of Hong Kong. Environ. Pollut. 96, 61–68. doi:10.1016/S0269-7491(97)00003-1

Cichon, M., Dubiec, A., 2005. Cell-mediated immunity predicts the probability of local recruitment in nestling blue tits. J. Evol. Biol. 18, 962–966. doi:10.1111/j.1420-9101.2005.00910.x

Clusella Trullas, S., van Wyk, J.H., Spotila, J.R., 2007. Thermal melanism in ectotherms. J. Therm. Biol. 32, 235–245. doi:10.1016/j.jtherbio.2007.01.013

Cook, L.M., 2000. Changing views on melanic moths. Biol. J. Linn. Soc. 69, 431–441. doi:10.1111/j.1095-8312.2000.tb01215.x

Cory-Slechta, D.A., Virgolini, M.B., Thiruchelvam, M., Weston, D.D., Bauter, M.R., 2004. Maternal stress modulates the effects of developmental lead exposure. Environ. Health Perspect. 112, 717–730. doi:10.1289/ehp.6481

Cosson, R.P., Amiard, J.-C., Amiard-Triquet, C., 1988. Trace elements in little egrets and flamingos of Camargue, France. Ecotoxicol. Environ. Saf. 15, 107–116. doi:10.1016/0147-6513(88)90047-4

Dauwe, L. Bervoets, R. Blust, M. Ee, T., 2002. Tissue levels of lead in experimentally exposed zebra finches (*Taeniopygia guttata*) with particular attention on the use of feathers as biomonitors. Arch. Environ. Contam. Toxicol. 42, 88–92. doi:10.1007/s002440010295

Dauwe, T., Eens, M., 2008. Melanin- and carotenoid-dependent signals of great tits (*Parus major*) relate differently to metal pollution. Naturwissenschaften 95, 969–973. doi:10.1007/s00114-008-0400-1

Dauwe, T., Janssens, E., Kempenaers, B., Eens, M., 2004. The effect of heavy metal exposure on egg size, eggshell thickness and the number of spermatozoa in blue tit *Parus caeruleus* eggs. Environ. Pollut. 129, 125–129. doi:10.1016/j.envpol.2003.09.028

Dibner, J.J., Knight, C.D., Kitchell, M.L., Atwell, C.A., Downs, A.C., Ivey, F.J., 1998. Early feeding and development of the immune system in neonatal poultry1. J. Appl. Poult. Res. 7, 425–436. doi:10.1093/japr/7.4.425

Dmowski, K., Gast, F., Müller, P., Wagner, G., 1984. Variability of cadmium and lead concentrations in bird feathers. Naturwissenschaften 71, 639–640.

Dotterud, L.K. are, Wilsgaard, T., Vorland, L.H., Falk, E.S., 2008. The effect of UVB radiation on skin microbiota in patients with atopic dermatitis and healthy controls. Int. J. Circumpolar Health 67.

Dubey, S., Roulin, A., 2014. Evolutionary and biomedical consequences of internal melanins. Pigment Cell Melanoma Res. 27, 327–338. doi:10.1111/pcmr.12231

Ducrest, A., Keller, L., Roulin, A., 2008. Pleiotropy in the melanocortin system, coloration and behavioural syndromes. Trends Ecol. Evol. 23, 502–510. doi:10.1016/j.tree.2008.06.001

Eeva, T., Ahola, M., Lehikoinen, E., 2009. Breeding performance of blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*) in a heavy metal polluted area. Environ. Pollut. 157, 3126–3131. doi:10.1016/j.envpol.2009.05.040

Eeva, T., Lehikoinen, E., 1996. Growth and mortality of nestling great tits (*Parus major*) and pied flycatchers (*Ficedula hypoleuca*) in a heavy metal pollution gradient. Oecologia 108, 631–639. doi:10.1007/BF00329036

Eeva, T., Sillanpää, S., Salminen, J.-P., Nikkinen, L., Tuominen, A., Toivonen, E., Pihlaja, K., Lehikoinen, E., 2008. Environmental pollution affects the plumage color of great tit nestlings through carotenoid availability. EcoHealth 5, 328–337. doi:10.1007/s10393-008-0184-y

El-Gazzar, R.M., Finelli, V.N., Boiano, J., Petering, H.G., 1978. Influence of dietary zinc on lead toxicity in rats. Toxicol. Lett. 1, 227–234. doi:10.1016/0378-4274(78)90053-X

Eraud, C., Jacquet, A., Faivre, B., 2009. Survival cost of an early immune soliciting in nature. Evolution 63, 1036–1043. doi:10.1111/j.1558-5646.2008.00540.x

Evans, G.W., Grace, C.I., Hahn, C., 1973. Homeostatic regulation of zinc absorption in the rat. Exp. Biol. Med. 143, 723–725. doi:10.3181/00379727-143-37400

Evans, M.R., Goldsmith, A.R., Norris, S.R.A., 2000. The effects of testosterone on antibody production and plumage coloration in male house sparrows (*Passer domesticus*). Behav. Ecol. Sociobiol. 47, 156–163. doi:10.1007/s002650050006

Franceschini, M.D., Lane, O.P., Evers, D.C., Reed, J.M., Hoskins, B., Romero, L.M., 2009. The corticosterone stress response and mercury contamination in free-living tree swallows, *Tachycineta bicolor*. Ecotoxicology 18, 514–521. doi:10.1007/s10646-009-0309-2

Frantz, A., Pottier, M.-A., Karimi, B., Corbel, H., Aubry, E., Haussy, C., Gasparini, J., Castrec-Rouelle, M., 2012. Contrasting levels of heavy metals in the feathers of urban pigeons from close habitats suggest limited movements at a restricted scale. Environ. Pollut. 168, 23–28. doi:10.1016/j.envpol.2012.04.003

Galván, I., Solano, F., 2009. The evolution of eu- and pheomelanic traits may respond to an economy of pigments related to environmental oxidative stress. Pigment Cell Melanoma Res. 22, 339–342. doi:10.1111/j.1755-148X.2009.00559.x

Gasparini, J., Jacquin, L., Laroucau, K., Vorimore, F., Aubry, E., Castrec-Rouëlle, M., Frantz, A., 2014. Relationships between metals exposure and epidemiological parameters of two pathogens in urban pigeons. Bull. Environ. Contam. Toxicol. 92, 208–212. doi:10.1007/s00128-013-1172-7

Giller, K.E., Witter, E., Mcgrath, S.P., 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biol. Biochem. 30, 1389–1414. doi:10.1016/S0038-0717(97)00270-8

Giraudeau, M., Mousel, M., Earl, S., McGraw, K., 2014. Parasites in the city: degree of urbanization predicts poxvirus and coccidian infections in house finches (*Haemorhous mexicanus*). PLoS ONE 9, e86747. doi:10.1371/journal.pone.0086747

Gochfeld, M., Saliva, J., Lesser, F., Shukla, T., Bertrand, D., Burger, J., 1991. Effects of color on cadmium and lead levels in avian contour feathers. Arch. Environ. Contam. Toxicol. 20, 523–526.

Godwin, H.A., 2001. The biological chemistry of lead. Curr. Opin. Chem. Biol. 5, 223–227. doi:10.1016/S1367-5931(00)00194-0

Goldstein, G., Flory, K.R., Browne, B.A., Majid, S., Ichida, J.M., Burtt Jr., E.H., 2004. Bacterial degradation of black and white feathers. The Auk 121, 656. doi:10.1642/0004-8038(2004)121[0656:BDOBAW]2.0.CO;2

Greenberg, S.A., Briemberg, H.R., 2004. A neurological and hematological syndrome associated with zinc excess and copper deficiency. J. Neurol. 251, 111–114. doi:10.1007/s00415-004-0263-0

Groothuis, T.G.., Schwabl, H., 2008. Hormone-mediated maternal effects in birds: mechanisms matter but what do we know of them? Philos. Trans. R. Soc. B Biol. Sci. 363, 1647–1661. doi:10.1098/rstb.2007.0007

Gulson, B., Mizon, K.J., Korsch, M.J., Howarth, D., Phillips, A., Hall, J., 1996. Impact on blood lead in children and adults following relocation from their source of exposure and contribution of skeletal tissue to blood lead. Bull. Environ. Contam. Toxicol. 56, 543–550. doi:10.1007/s001289900078

Gunderson, A.R., Forsyth, M.H., Swaddle, J.P., 2009. Evidence that plumage bacteria influence feather coloration and body condition of eastern bluebirds *Sialia sialis*. J. Avian Biol. 40, 440–447. doi:10.1111/j.1600-048X.2008.04650.x

Gunderson, A.R., Frame, A.M., Swaddle, J.P., Forsyth, M.H., 2008. Resistance of melanized feathers to bacterial degradation: is it really so black and white? J. Avian Biol. 39, 539–545.

Hanssen, S.A., Hasselquist, D., Folstad, I., Erikstad, K.E., 2004. Costs of immunity: immune responsiveness reduces survival in a vertebrate. Proc. R. Soc. B Biol. Sci. 271, 925–930. doi:10.1098/rspb.2004.2678

Hargitai, R., Prechl, J., TöRöK, J., 2006. Maternal immunoglobulin concentration in collared flycatcher (*Ficedula albicollis*) eggs in relation to parental quality and laying order. Funct. Ecol. 20, 829–838. doi:10.1111/j.1365-2435.2006.01171.x

Hasselquist, D., Nilsson, J.-A., 2009. Maternal transfer of antibodies in vertebrates: trans-generational effects on offspring immunity. Philos. Trans. R. Soc. B Biol. Sci. 364, 51–60. doi:10.1098/rstb.2008.0137

Heller, E.D., Leitner, H., Drabkin, N., Melamed, D., 1990. Passive immunisation of chicks against *Escherichia coli*. Avian Pathol. 19, 345–354. doi:10.1080/03079459008418685

Herring, G., Ackerman, J.T., Herzog, M.P., 2012. Mercury exposure may suppress baseline corticosterone levels in juvenile birds. Environ. Sci. Technol. 46, 6339–6346. doi:10.1021/es300668c

Hildebrand, F., Nguyen, T.L., Brinkman, B., Yunta, R.G., Cauwe, B., Vandenabeele, P., Liston, A., Raes, J., 2013. Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. Genome Biol 14, R4.

Hill, H.Z., Hill, G.J., 2000. UVA, Pheomelanin and the carcinogenesis of melanoma. Pigment Cell Res. 13, 140–144. doi:10.1034/j.1600-0749.13.s8.25.x

Hoff Brait, C.H., Antoniosi Filho, N.R., 2011. Use of feathers of feral pigeons (*Columba livia*) as a technique for metal quantification and environmental monitoring. Environ. Monit. Assess. 179, 457–467. doi:10.1007/s10661-010-1748-1

Hong, L., Simon, J.D., 2007. Current understanding of the binding sites, capacity, affinity, and biological significance of metals in melanin. J. Phys. Chem. B 111, 7938–7947. doi:10.1021/jp071439h

Horrocks, N.P.C., Irene Tieleman, B., Matson, K.D., 2011. A simple assay for measurement of ovotransferrin - a marker of inflammation and infection in birds: Ovotransferrin assay for plasma from wild birds. Methods Ecol. Evol. 2, 518–526. doi:10.1111/j.2041-210X.2011.00096.x

Ito, S., Wakamatsu, K., Ozeki, H., 2000. Chemical analysis of melanins and its application to the study of the regulation of melanogenesis. Pigment Cell Res. 13, 103–109. doi:10.1034/j.1600-0749.13.s8.19.x

Jacob, S., Immer, A., Leclaire, S., Parthuisot, N., Ducamp, C., Espinasse, G., Heeb, P., 2014. Uropygial gland size and composition varies according to experimentally modified microbiome in Great tits. BMC Evol. Biol. 14, 134.

Jacquin, L., Haussy, C., Bertin, C., Laroucau, K., Gasparini, J., 2013. Darker female pigeons transmit more specific antibodies to their eggs than do paler ones. Biol. J. Linn. Soc. 108, 647–657.

Jacquin, L., Lenouvel, P., Haussy, C., Ducatez, S., Gasparini, J., 2011. Melanin-based coloration is related to parasite intensity and cellular immune response in an urban free living bird: the feral pigeon *Columba livia*. J. Avian Biol. 42, 11–15. doi:10.1111/j.1600-048X.2010.05120.x

Jacquin, L., Récapet, C., Prévot-Julliard, A.-C., Leboucher, G., Lenouvel, P., Erin, N., Corbel, H., Frantz, A., Gasparini, J., 2013. A potential role for parasites in the maintenance of color polymorphism in urban birds. Oecologia 173, 1089–1099. doi:10.1007/s00442-013-2663-2

Jarup, L., 2003. Hazards of heavy metal contamination. Br. Med. Bull. 68, 167–182. doi:10.1093/bmb/ldg032

Johnston, R., Janiga, M., 1995. Feral pigeons. Oxford University Press.

Kekkonen, J., Hanski, I.K., Väisänen, R.A., Brommer, J.E., 2012. Levels of heavy metals in house sparrows (*Passer domesticus*) from urban and rural habitats of southern Finland. Ornis Fenn. 89.

Kim, E.Y., Goto, R., Tanabe, S., Tanaka, H., Tatsukawa, R., 1998. Distribution of 14 elements in tissues and organs of oceanic seabirds. Arch. Environ. Contam. Toxicol. 35, 638–645. doi:10.1007/s002449900426

Koivula, M.J., Eeva, T., 2010. Metal-related oxidative stress in birds. Environ. Pollut. 158, 2359–2370. doi:10.1016/j.envpol.2010.03.013

Larsson, B., Tjälve, H., 1978. Studies on the melanin-affinity of metal ions. Acta Physiol. Scand. 104, 479–484. doi:10.1111/j.1748-1716.1978.tb06303.x

Leclaire, S., Pierret, P., Chatelain, M., Gasparini, J., 2014. Feather bacterial load affects plumage condition, iridescent color, and investment in preening in pigeons. Behav. Ecol. 25, 1192–1198. doi:10.1093/beheco/aru109

Liu, Y., Hong, L., Kempf, V.R., Wakamatsu, K., Ito, S., Simon, J.D., 2004. Ion-exchange and adsorption of Fe (III) by sepia melanin. Pigment Cell Res. 17, 262–269.

Lochmiller, R.L., Deerenberg, C., 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88, 87–98. doi:10.1034/j.1600-0706.2000.880110.x

Lucia, M., André, J.-M., Gontier, K., Diot, N., Veiga, J., Davail, S., 2010. Trace element concentrations (mercury, cadmium, copper, zinc, lead, aluminium, nickel, arsenic, and selenium) in some aquatic birds of the southwest atlantic coast of France. Arch. Environ. Contam. Toxicol. 58, 844–853. doi:10.1007/s00244-009-9393-9

Maas, S., Scheifler, R., Benslama, M., Crini, N., Lucot, E., Brahmia, Z., Benyacoub, S., Giraudoux, P., 2010. Spatial distribution of heavy metal concentrations in urban, suburban and agricultural soils in a Mediterranean city of Algeria. Environ. Pollut. 158, 2294–2301. doi:10.1016/j.envpol.2010.02.001

Manta, D.S., Angelone, M., Bellanca, A., Neri, R., Sprovieri, M., 2002. Heavy metals in urban soils: a case study from the city of Palermo (Sicily), Italy. Sci. Total Environ. 300, 229–243. doi:10.1016/S0048-9697(02)00273-5

Marchetti, C., 2003. Molecular targets of lead in brain neurotoxicity. Neurotox. Res. 5, 221–235. doi:10.1007/BF03033142

Mauck, R.A., Matson, K.D., Philipsborn, J., Ricklefs, R.E., 2005. Increase in the constitutive innate humoral immune system in Leach's storm-petrel (*Oceanodroma leucorhoa*) chicks is negatively

correlated with growth rate. Funct. Ecol. 19, 1001–1007. doi:10.1111/j.1365-2435.2005.01060.x

McGraw, K.J., 2006. Dietary mineral content influences the expression of melanin-based ornamental coloration. Behav. Ecol. 18, 137–142. doi:10.1093/beheco/arl059

McGraw, K.J., 2003. Melanins, metals, and mate quality. Oikos 402–406.

McKinney, M.L., 2008. Effects of urbanization on species richness: A review of plants and animals. Urban Ecosyst. 11, 161–176. doi:10.1007/s11252-007-0045-4

Meister, A., 1992. On the antioxidant effects of ascorbic acid and glutathione. Biochem. Pharmacol. 44, 1905–1915. doi:10.1016/0006-2952(92)90091-V

Mishra, K.P., 2009. Lead exposure and its impact on immune system: A review. Toxicol. In Vitro 23, 969–972. doi:10.1016/j.tiv.2009.06.014

Møller, A.P., Saino, N., 2004. Immune response and survival. Oikos 104, 299–304. doi:10.1111/j.0030-1299.2004.12844.x

Moreno-Rueda, G., 2010. Uropygial gland size correlates with feather holes, body condition and wingbar size in the house sparrow *Passer domesticus*. J. Avian Biol. 41, 229–236. doi:10.1111/j.1600-048X.2009.04859.x

Mueller, S., Saunier, K., Hanisch, C., Norin, E., Alm, L., Midtvedt, T., Cresci, A., Silvi, S., Orpianesi, C., Verdenelli, M.C., Clavel, T., Koebnick, C., Zunft, H.-J.F., Dore, J., Blaut, M., 2006. Differences in fecal microbiota in different european study populations in relation to age, gender, and country: a cross-sectional study. Appl. Environ. Microbiol. 72, 1027–1033. doi:10.1128/AEM.72.2.1027-1033.2006

Naber, E.C., 1979. The effect of nutrition on the composition of eggs. Poult. Sci. 58, 518–528. doi:10.3382/ps.0580518

Nam, D.-H., Lee, D.-P., Koo, T.-H., 2004. Monitoring for lead pollution using feathers of feral pigeons (*Columba livia*) from Korea. Environ. Monit. Assess. 95, 13–22. doi:10.1023/B:EMAS.0000029898.28393.30

Neil, K., Wu, J., 2006. Effects of urbanization on plant flowering phenology: A review. Urban Ecosyst. 9, 243–257. doi:10.1007/s11252-006-9354-2

Niecke, M., Heid, M., Krüger, A., 1999. Correlations between melanin pigmentation and element concentration in feathers of white-tailed eagles (*Haliaeetus albicilla*). J. Für Ornithol. 140, 355–362.

Nriagu, J.O., 1989. A global assessment of natural sources of atmospheric trace metals. Nature 338, 47–49.

Nriagu, J.O., 1979. Global inventory of natural and anthropogenic emissions of trace metals to the atmosphere. Nature 279, 409–411. doi:10.1038/279409a0

Nuran Ercal, B.S.P., Hande Gurer-Orhan, B.S.P., Nukhet Aykin-Burns, B.S.P., 2001. Toxic metals and oxidative stress Part I: mechanisms involved in me-tal induced oxidative damage. Curr. Top. Med. Chem. 1, 529–539. doi:10.2174/1568026013394831

Obukhova, N., 2007. Polymorphism and phene geography of the blue rock pigeon in Europe. Russ J Genet 43, 492–501.

Partecke, J., Van't Hof, T., Gwinner, E., 2004. Differences in the timing of reproduction between urban and forest European blackbirds (*Turdus merula*): result of phenotypic flexibility or genetic differences? Proc. R. Soc. B Biol. Sci. 271, 1995–2001. doi:10.1098/rspb.2004.2821

Piault, R., Gasparini, J., Bize, P., Paulet, M., McGraw, K.J., Roulin, A., 2008. Experimental support for the makeup hypothesis in nestling tawny owls (*Strix aluco*). Behav. Ecol. 19, 703–709. doi:10.1093/beheco/arm152

Pihlaja, M., Siitari, H., Alatalo, R.V., 2006. Maternal antibodies in a wild altricial bird: effects on offspring immunity, growth and survival: Maternal antibodies in an altricial bird. J. Anim. Ecol. 75, 1154–1164. doi:10.1111/j.1365-2656.2006.01136.x

Pohanka, M., Hubalek, J., Beklova, M., Vrba, R., Kizek, R., 2012. Metallomics study of lead-protein interactions in albumen by electrochemical and electrophoretic methods. Int J Electrochem Sci 7, 943–964.

Pongratz, R., Heumann, K.G., 1999. Production of methylated mercury, lead, and cadmium by marine bacteria as a significant natural source for atmospheric heavy metals in polar regions. Chemosphere 39, 89–102. doi:10.1016/S0045-6535(98)00591-8

Prasad, A.S., 2009. Zinc: role in immunity, oxidative stress and chronic inflammation: Curr. Opin. Clin. Nutr. Metab. Care 12, 646–652. doi:10.1097/MCO.0b013e3283312956

Prasad, A.S., 1998. Zinc and immunity. Mol. Cell. Biochem. 188, 63–69.

Récapet, C., Dauphin, L., Jacquin, L., Gasparini, J., Prévot-Julliard, A.-C., 2013. Eumelanin-based colouration reflects local survival of juvenile feral pigeons in an urban pigeon house. J. Avian Biol. 44, 583–590. doi:10.1111/j.1600-048X.2013.00087.x

Redig, P.T., Lawler, E.M., Schwartz, S., Dunnette, J.L., Stephenson, B., Duke, G.E., 1991. Effects of chronic exposure to sublethal concentrations of lead acetate on heme synthesis and immune function in red-tailed hawks. Arch. Environ. Contam. Toxicol. 21, 72–77.

Roulin, A., 2004. The evolution, maintenance and adaptive function of genetic colour polymorphism in birds. Biol. Rev. 79, 815–848.

Roulin, A., Almasi, B., Meichtry-Stier, K.S., Jenni, L., 2011. Eumelanin- and pheomelanin-based colour advertise resistance to oxidative stress in opposite ways: Melanin colour and resistance to oxidative stress. J. Evol. Biol. 24, 2241–2247. doi:10.1111/j.1420-9101.2011.02353.x

Roux, K.E., Marra, P.P., 2007. The presence and impact of environmental lead in passerine birds along an urban to rural land use gradient. Arch. Environ. Contam. Toxicol. 53, 261–268. doi:10.1007/s00244-006-0174-4

Ruiz-de-Castañeda, R., Vela, A.I., Lobato, E., Briones, V., Moreno, J., 2011. Bacterial loads on eggshells of the pied flycatcher: environmental and maternal factors. The Condor 113, 200–208. doi:10.1525/cond.2011.100035

Rutz, C., 2012. Predator fitness increases with selectivity for odd prey. Curr. Biol. 22, 820–824. doi:10.1016/j.cub.2012.03.028

Saino, N., Dall'Ara, P., Martinelli, R., Møller, A.P., 2002. Early maternal effects and antibacterial immune factors in the eggs, nestlings and adults of the barn swallow. J. Evol. Biol. 15, 735–743.

Samokhvalov, A., Hong, L., Liu, Y., Garguilo, J., Nemanich, R.J., Edwards, G.S., Simon, J.D., 2007. Oxidation potentials of human eumelanosomes and pheomelanosomes¶. Photochem. Photobiol. 81, 145–148. doi:10.1111/j.1751-1097.2005.tb01533.x

Scheifler, R., Cœurdassier, M., Morilhat, C., Bernard, N., Faivre, B., Flicoteaux, P., Giraudoux, P., Noël, M., Piotte, P., Rieffel, D., de Vaufleury, A., Badot, P.-M., 2006. Lead concentrations in feathers and blood of common blackbirds (*Turdus merula*) and in earthworms inhabiting unpolluted and moderately polluted urban areas. Sci. Total Environ. 371, 197–205. doi:10.1016/j.scitotenv.2006.09.011

Schulz, J.H., Millspaugh, J.J., Bermudez, A.J., Gao, X., Bonnot, T.W., Britt, L.G., Paine, M., 2006. Acute lead toxicosis in mourning doves. J. Wildl. Manag. 70, 413–421. doi:10.2193/0022-541X(2006)70[413:ALTIMD]2.0.CO;2

Schwartz, J., Landrigan, P.J., Baker, E.L., Orenstein, W.A., von Lindern, I.H., 1990. Lead-induced anemia: dose-response relationships and evidence for a threshold. Am. J. Public Health 80, 165–168. doi:10.2105/AJPH.80.2.165

Sens, E.J., Dauwe, T., Pinxten, R., Eens, M., 2003. Breeding performance of great tits (*Parus major*) along a gradient of heavy metal pollution. Environ. Toxicol. Chem. 22, 1140–1145.

Seto, K.C., Guneralp, B., Hutyra, L.R., 2012. Global forecasts of urban expansion to 2030 and direct impacts on biodiversity and carbon pools. Proc. Natl. Acad. Sci. 109, 16083–16088. doi:10.1073/pnas.1211658109

Shawkey, M.D., Pillai, S.R., Hill, G.E., Siefferman, L.M., Roberts, S.R., 2007. Bacteria as an agent for change in structural plumage color: correlational and experimental evidence. Am. Nat. 169, S112–S121. doi:10.1086/510100

Shaw, L.M., Chamberlain, D., Evans, M., 2008. The house sparrow *Passer domesticus* in urban areas: reviewing a possible link between post-decline distribution and human socioeconomic status. J. Ornithol. 149, 293–299. doi:10.1007/s10336-008-0285-y

Snoeijs, T., Dauwe, T., Pinxten, R., Darras, V., Arckens, L., Eens, M., 2005. The combined effect of lead exposure and high or low dietary calcium on health and immunocompetence in the zebra finch. Environ. Pollut. 134, 123–132. doi:10.1016/j.envpol.2004.07.009

Snoeijs, T., Dauwe, T., Pinxten, R., Vandesande, F., Eens, M., 2004. Heavy metal exposure affects the humoral immune response in a free-living small songbird, the great tit (*Parus major*). Arch. Environ. Contam. Toxicol. 46. doi:10.1007/s00244-003-2195-6

Toscano, C.D., Guilarte, T.R., 2005. Lead neurotoxicity: From exposure to molecular effects. Brain Res. Rev. 49, 529–554. doi:10.1016/j.brainresrev.2005.02.004

Valenti, P., Antonini, G., Von Hunolstein, C., Visca, P., Orsi, N., Antonini, E., 1983. Studies of the antimicrobial activity of ovotransferrin. Int. J. Tissue React. 5, 97–105.

Valenti, P., Visca, P., Antonini, G., Orsi, N., 1985. Antifungal activity of ovotransferrin towards genus Candida. Mycopathologia 89, 169–175. doi:10.1007/BF00447027

Vyskočil, A., Fiala, Z., Ettlerová, E., Tenjnorová, I., 1990. Influence of chronic lead exposure on hormone levels in developing rats. J. Appl. Toxicol. 10, 301–302.

Wayland, M., 2002. Immune function, stress response, and body condition in arctic-breeding common eiders in relation to cadmium, mercury, and selenium concentrations. Environ. Res. 90, 47–60. doi:10.1006/enrs.2002.4384

Williamson, J.M., Boettcher, B., Meister, A., 1982. Intracellular cysteine delivery system that protects against toxicity by promoting glutathione synthesis. Proc. Natl. Acad. Sci. U. S. A. 79, 6246–6249.

Wirth, J.J., Fraker, P.J., Kierszenbaum, F., 1989. Zinc requirement for macrophage function: effect of zinc deficiency on uptake and killing of a protozoan parasite. Immunology 68, 114.

Yadav, S.K., 2010. Heavy metals toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. South Afr. J. Bot. 76, 167–179. doi:10.1016/j.sajb.2009.10.007

Youssef, S.A.., El-Sanousi, A.., Afifi, N.., El-Brawy, A.M.., 1996. Effect of subclinical lead toxicity on the immune response of chickens to Newcastle disease virus vaccine. Res. Vet. Sci. 60, 13–16. doi:10.1016/S0034-5288(96)90123-X

Zduniak, P., Surmacki, A., Erciyas-Yavuz, K., Chudzińska, M., Barałkiewicz, D., 2014. Are there different requirements for trace elements in eumelanin- and pheomelanin-based color production? A case study of two passerine species. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 175, 96–101. doi:10.1016/j.cbpa.2014.05.01

Behavioral Ecology Advance Access published July 6, 2014

Behavioral Ecology

The official journal of the ISBE International Society for Behavioral Ecology

Behavioral Ecology (2014), 00(00), 1–7. doi:10.1093/beheco/aru109

Original Article

Feather bacterial load affects plumage condition, iridescent color, and investment in preening in pigeons

Sarah Leclaire, Pauline Pierret, Marion Chatelain, and Julien Gasparini

Laboratoire Ecologie et Evolution, UMR 7625, UPMC CNRS ENS, Université Pierre et Marie Curie, 7 quai St. Bernard, 75252 Paris, France

Received 26 March 2014; revised 26 May 2014; accepted 27 May 2014.

Feathers are inhabited by numerous bacteria, some of them being able to degrade feathers, and thus potentially alter thermoregulation and visual communication. To limit the negative effects of feather bacteria on fitness, birds have therefore evolved antimicrobial defense mechanisms, including preening feathers with secretions of the preen gland. However, whether feather bacteria can alter feather condition and color signaling in vivo, and thus whether birds adjust their investment in preening according to feather bacterial load, has barely been investigated. Here, we experimentally decreased and increased feather bacterial load on captive feral pigeons *Columba livia* and investigated the effects on plumage characteristics and investment in preening. We found that birds of both sexes had a plumage in higher condition and invested less in preen secretion quantity and preening behavior when feather bacterial load was lower. It suggests that preen secretions may be used by pigeons to limit feather degradation by bacteria, but as they are probably costly to produce, their quantity is adjusted depending on feather bacteria load. Birds with lower bacteria load on feathers had brighter iridescent neck feathers, suggesting that feather bacteria may play an important role in the evolution of the signaling function of iridescent color in pigeons. Altogether, our study provides the first experimental evidence for in vivo effects of feather bacteria on plumage degradation and coloration and suggests that preening is an inducible antibacterial defense.

Key words: **bacteria, birds, iridescence, plumage, preen oil.**

INTRODUCTION

Bacteria are fundamental associates of animal bodies living in digestive, respiratory, and reproductive tracts. Bacteria live not just within but also on the surface of bodies, in skin, and feathers (Tannock 1995; Burtt and Ichida 1999; Shawkey et al. 2003). Some of these bacteria are opportunistic pathogens (Scott 2001; Cogen et al. 2008), while others are part of the normal microflora (Tannock 1995). Recently, several studies have highlighted the potential influence of bacteria on animal behavior and communication (Archie et al. 2007; Sharon et al. 2010; Ezenwa et al. 2012). However, beyond the effects associated with bacterial infection (Hart 1988), we understand little about bacteria's more routine contribution to host behavior and life history traits.

In birds, a small subset of feather bacteria is detrimental to the bird by degrading keratin (i.e., keratinolytic bacteria) and causing damage to feathers (Burtt and Ichida 1999). By breaking down the structures of feathers, feather-degrading bacteria may reduce bird fitness via the alteration of thermoregulation, flight, and signaling (Swaddle et al. 1996; Clayton 1999; Shawkey et al. 2007). For instance, structural parameters of feathers may determine their water repellency (Rijke 1970; Giraudeau et al. 2010; Eliason and Shawkey 2011), an important component of thermoregulation, and flight efficiency. Feather degradation may also reduce feather coloration, as feather microstructures or pigments are consumed or modified through microbial action (Shawkey and Hill 2004). Accordingly, in vitro experiments have shown that feather-degrading bacteria brighten structurally colored feathers in male eastern bluebirds *Sialia sialis* (Shawkey et al. 2007). However, experimental in vivo work on the effects of feather bacteria on plumage has rarely been done. The only experimental study on live birds has shown no change in feather damage after inoculation with one species of feather-degrading bacteria (Cristol et al. 2005). Given the complexity of plumage bacterial communities, more in vivo experiments are required to test for the impact of plumage bacterial communities on feather condition and coloration (Gunderson 2008).

Moreover, if maintaining feather condition and coloration is important in term of fitness, birds must have evolved a number of antibacterial defenses (Gunderson 2008). For instance, the deposition of melanin pigments in feathers can constitute such adaptation

Address correspondence to S. Leclaire. E-mail: sarah.leclaire@free.fr.

by increasing feathers resistance to bacterial degradation. Several behaviors, such as sunbathing (as sunlight may destroy bacteria), molting, or preening may also constitute adaptation to plumage bacterial communities (Gunderson 2008). During preening, birds deposit oily secretions of the preen gland (also called uropygial gland) onto the plumage. These oily secretions have numerous properties (Jacob and Ziswiler 1982; Hagelin and Jones 2007), one of which is to protect feathers against feather-degrading bacteria (Shawkey et al. 2003). In vitro experiments have shown that preen secretions of several species inhibit the growth of isolated bacteria (Shawkey et al. 2003; Reneerkens et al. 2008). Furthermore, in house sparrows *Passer domesticus*, preen gland removal led to increased load of feather bacteria (Czirják et al. 2013) and, in barn Swallows *Hirundo rustica*, the abundance of feather-degrading bacteria decreased with increasing size of the uropygial gland (Møller et al. 2009). Furthermore, if preen secretions or preening are costly, birds are expected to invest in them only when bacterial load is high (i.e., induced defense; Harvell 1990; Tollrian and Harvell 1999).

Here, we experimentally increased and decreased bacterial load on the plumage of captive feral pigeons *Columba livia* to test whether load of feather bacteria affects plumage condition (quality, water repellency efficiency, and iridescent color) and investment in preening (preen secretion quantity and preening behavior).

MATERIALS AND METHODS

Experimental design

In March–May 2013, 80 feral pigeons (43 females and 37 males) were captured at different locations in Paris, France. They were kept in 6 outdoor aviaries at the CEREEP field station (Centre de recherche en Ecologie Expérimentale et Prédictive – Ecotron Ile-de-France, UMS 3194, Saint Pierre lès Nemours, France) in similar conditions and fed ad libitum with a mix of maize, wheat and peas, and mineral supplements. Birds were kept in captivity for ca. 2 months for acclimation to obtain naturally representative pigeon physiology and behavior. After acclimation, birds were assigned to treatment (BACT−: decreased feather bacterial load, BACT+: increased feather bacterial load, and CO: control treatment), and they were weighed to the nearest g, wing length was measured to the nearest mm, and melanin-based color morph was recorded. Feral pigeons display a continuous variation in eumelanin-based coloration from white to black that display differences in several life history traits (Jacquin et al. 2011; 2013). Therefore, we equally distributed eumelanin-based coloration of pigeons among treatments (Kruskal–Wallis test: $H_3 = 0.65$, $P = 0.89$). We did the same for body mass (linear model: $F_{2,77} = 0.45$, $P = 0.64$) and body condition (linear model: $F_{2,77} = 1.10$, $P = 0.34$). Birds were weighed at day 15, day 28, day 42, day 56, and day 70 after onset of treatment.

In the BACT− treatment, birds from 2 aviaries ($n = 14$ females and 13 males) were sprayed twice a week with 0.02% chlorhexidine (Hibitane Irrigation®, MSD) in saline solution (0.9% NaCl solution). Chlorhexidine is an antiseptic, frequently used as a topical antiseptic skin scrub and topical disinfectant of wounds in hospitals and veterinary clinics. In the CO treatment, birds from 2 aviaries ($n = 14$ females and 12 males) were sprayed twice a week with saline solution. In the BACT+ treatment, birds from 2 aviaries ($n = 15$ females and 12 males) were sprayed twice a week with freshly cultivated bacteria in saline solution. Freshly cultivated bacteria came from feather bacteria sampled from Parisian feral pigeons and cultivated on Tryptic Soy Agar (TSA) plates and feather meal agar (FMA) plates. TSA allows the growth of both keratinolytic and non-keratinolytic bacteria, while FMA allows the growth of keratinolytic bacteria only. We used both agar media to ensure the inoculation of keratinolytic bacteria in BACT+ birds. Each day of treatment, a total of 1.5 L of solution per aviary was used to spray birds. Birds of the same aviary got the same treatment to avoid potential transmission of the treatment between birds by social interactions.

We checked the effect of treatment on feather bacterial load by cultivating feathers bacteria on whole flora agar slides (plate count agar + triphenyltetrazolium chloride + neutralizing dip slides; VWR BDH Prolabo), every fortnight for 2.5 months ($n = 6$ control date). Slides were pressed for 10 s onto the back feathers of 4 random birds of each treatment and then incubated for 24–48 h at 37 °C. Feather bacterial load was expressed as the number of bacterial colonies per slide.

Iridescent feather color

After 1.5 months of treatment, 5–10 feathers from the left side of the neck were cut at the base and stored at −20 °C until analyses. Pigeons display 2 kinds of iridescent neck feathers, the green and purple feathers, which show color changes in opposite ways when reflection angles vary (McGraw 2004; Yin et al. 2006; Yoshioka et al. 2007). Here, we focused on feathers that appeared green in color to the human eye at normal incidence. Neck feathers were mounted on a black velvet card and color was measured with a reflectance spectrometer (Ocean Optics USB2000), a Xenon light source (Ocean Optics PX-2), and a 200-µm fiber optic reflectance probe. The probe was inserted in a black tube in a way that the probe incidence angle was 90°, but that the probe could be slightly orientated to maximize reflectance. Reflectance was measured using SpectraSuite software (Ocean Optics, Inc.) and in relation to a dark and a white (Spectralon®, Labsphere) standard. The spectrometer was calibrated between each 15 measurements. Reflectance measurements were done blind according to treatments. For each bird, feather color was measured 3 times and the 3 spectra were then averaged to make a single measurement.

Iridescent neck feathers that appeared green in color to the human eye exhibited 3 full reflectance peaks (McGraw 2004), one in the UVB range (average $\lambda_{max} = 357 \pm 1$ nm), one in the violet range (average $\lambda_{max} = 431 \pm 2$ nm), and one in the green range (average $\lambda_{max} = 548 \pm 2$ nm). Two additional peaks—one in the UVB range and one in the red range—were frequently truncated when considering the 300–700 nm range (Figure 1).

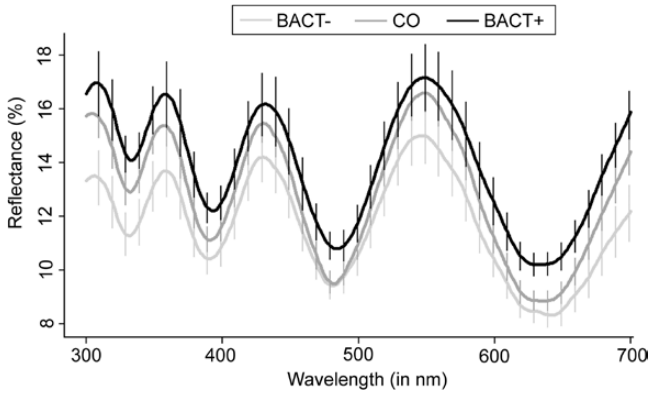


Figure 1
Mean reflectance spectra ± SE of iridescent neck green feathers in BACT−, CO, and BACT+ pigeons.

To assess whether birds from the 3 different treatments appeared dissimilar to their conspecifics, we modeled spectral sensitivities of pigeons and computed photoreceptor responses (quantum catches) and brightness (luminance) using models developed for the tetrachromatic visual system of birds. We modeled spectral sensitivities of cone photoreceptors between 300 and 700 nm according to the model of Hart and Vorobyev (2005), using oil droplet and single cone photoreceptor spectral parameters of pigeons published by Bowmaker et al. (1997), and the ocular media transmittance of pigeons measured by Lind et al. (2014). We computed photoreceptor responses and brightness using the Endler and Mielke's model (2005). We used the sum of the 2 longest-wavelength cones as the sensitivity data to calculate achromatic cone stimulation. We chose the standard illuminants D65 as a representative spectrum for open habitat midday ambient light. All spectral analyses were conducted with the "pavo" package (Maia et al. 2013) in R statistical software (R Development Core Team 2014).

Repeatability (intraclass coefficient) of the 4 quantum catches and brightness within birds was measured using the "ICC" package in R (Wolak et al. 2012) and was mean (95% confidence interval): 0.52 (0.39–0.64), 0.68 (0.57–0.77), 0.59 (0.46–0.70), 0.61 (0.49–0.72), and 0.45 (0.31–0.58), respectively.

Plumage condition and hydrophobicity

In order to measure plumage condition, we used a similar method as Moyer et al. (2003). After 2 months of treatment, around 5 feathers from the lower back of the birds were collected by cutting them at the base and stored in plastic bags until analyses. Plumage quality was then scored from 1 to 5 (1 = very poor condition, 2 = poor condition, 3 = fair, 4 = good, and 5 = very good; Figure 2), blind to treatment. Condition was scored twice independently. As scores were repeatable ($r = 0.60$, $P < 0.0001$; ICC = 0.61), analyses of plumage condition was performed using average values.

After 3 months of treatment, plumage water retention efficiency was tested using the protocol described in Giraudeau et al. (2010). Briefly, we measured the bird's dry weight (to the nearest 0.1 g), then we submerged the birds into a water bath (except the head) for an exact duration of 5 s, by gently holding them in a way that they could not open their wings. After 5 s, we lifted the birds from the water and waited for 5 s before the second weighing to let the extra water run-off of the surface of plumage. The difference between the 2 weights indicated the amount of water retained by the plumage and provided a measure of plumage wetness. Birds were kept without any access to water during 2 h before the test to be sure that their plumage was dry at the moment of the measurement.

Preen secretion and preening behavior

After 2.5 months of treatment, preen secretions were collected by gently pressing the gland and collecting the exudates in 20 µL glass capillaries. The gland was pressed until no more secretion went out. Secretion quantity into the capillary was measured as the total capillary length filled with secretion (to the nearest 0.5 mm) and then converted to µL. One bird had no gland, and another bird had a gland orifice which seemed to be blocked. An abnormal ball of preen oil appeared within the preen duct but no oil exuded when the gland was pressed. These 2 birds were excluded from the analyses on preen secretion quantity, preening behavior, and water repellency efficiency.

Preening behaviors were observed for 46 days after onset of treatment. Each bird was observed for 5 min once or twice a week ($n = 790$ focal sessions). Focal birds were randomly chosen but by alternating between treatments. Furthermore, when a bird was observed only once a week, it was observed twice in the following week. For each bird, we calculated the average percentage of time spent preening per 5-min observation. Birds were excluded from the analyses once they had laid eggs ($n = 14$ birds; 46 focal sessions).

Statistical analyses

The effect of treatment on log-transformed feather bacterial load was tested using a linear model with treatment and date as fixed effects. The effects of treatment on preen secretion quantity, percentage of time spent preening, log-transformed plumage water retention, plumage condition, and color (each of the 4 receptor

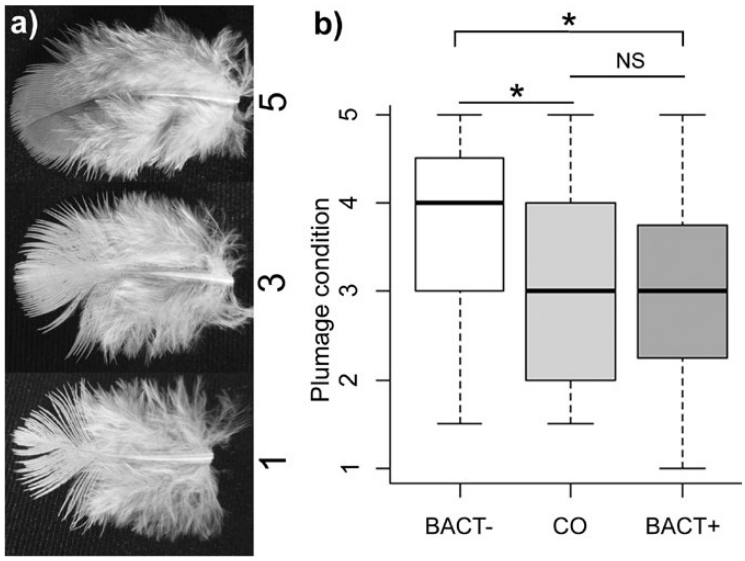


Figure 2
(a) Examples of back feather condition (1: very poor condition, 3: fair condition, and 5: very good condition). (b) Boxplot of back feather condition in BACT−, CO, and BACT+ pigeons. *$P < 0.05$, NS: $P > 0.05$.

quantum catches, and brightness) were tested using linear mixed models, with treatment, sex, color morph, and all double interactions as fixed effects. Body condition, measured as the residual of a regression between body mass and wing length, was also added as a fixed effect in the analyses. We did not use tarsus length as the index of body size as it strongly depended on the observer ($P < 0.0001$) and was not measured for all birds. In contrast, wing length was measured for all experimental birds and did not depend on the observer ($P > 0.10$). Wing length was slightly correlated with tarsus length (Pearson correlation: $r = 0.27$, $P = 0.017$). We began by including the aviary identity as a random factor in the models. However, it was significant in none of the analyses, and we excluded it from the models. When the treatment was significant, we used Tukey's tests to determine the treatments that significantly differ from each other. The effects of treatment on body condition was tested using linear mixed models with treatment, sex, morph and their interactions, and date as fixed effects. Bird identity was included as a random effect. Body condition at onset of treatment was added as a covariate. Model selection was performed by backward dropping nonsignificant terms (unless they appeared in higher order interaction terms) using a stepwise elimination procedure.

All statistical tests were conducted with SAS, version 9.1 (SAS Institute, Cary, NC). We used 2-tailed Type 3 tests for fixed effects with significance level set at $\alpha = 0.05$.

RESULTS

Feather bacterial load varied among treatments ($F_{2,64} = 29.89$, $P < 0.0001$; Figure 3). BACT+ birds had higher bacterial load than BACT− birds ($P < 0.0001$), while CO birds had intermediate bacterial load (CO vs. BACT+: $P < 0.0001$ and CO vs. BACT−: $P = 0.037$; Figure 3).

Body condition did not differ among treatments ($F_{2,73} = 1.56$, $P = 0.22$), but it depended upon the interaction between sex and color morph ($F_{1,75} = 5.73$, $P = 0.019$). Darker females are in better condition than paler females ($F_{1,40} = 8.42$, $P = 0.006$), while body condition did not differ among color morphs in males ($F_{1,34} = 0.26$, $P = 0.61$).

Quantity of preen secretion differed among treatments ($F_{2,74} = 6.88$, $P = 0.0018$; Figure 4a). BACT− birds had a lower quantity of preen secretion than CO and BACT+ birds ($P = 0.03$ and $P = 0.002$; Figure 4a), while BACT+ and CO birds had a similar quantity of secretion ($P = 0.57$; Figure 4a). Birds in better condition had a lower quantity of preen secretion than birds in lower condition ($F_{1,74} = 5.83$, $P = 0.018$).

Duration of preening differed among treatments ($F_{2,73} = 4.14$, $P = 0.02$; Figure 4b). BACT− birds preened less often than BACT+ birds ($P = 0.018$; Figure 4b). Duration of preening of CO birds was intermediate, but not significantly different from that of BACT− ($P = 0.13$) and BACT+ birds ($P = 0.72$; Figure 4b). Males preened for longer than females ($F_{2,73} = 4.49$, $P = 0.038$), and darker pigeons preened for longer than paler pigeons ($F_{2,73} = 5.89$, $P = 0.018$).

Condition of back feathers differed among treatments ($F_{2,76} = 6.81$, $P = 0.0019$; Figure 2b). BACT− birds had back feathers in higher condition than CO and BACT+ birds ($P = 0.045$ and $P = 0.0016$, respectively; Figure 2b), while feather condition of BACT+ birds did not significantly differ from that of CO birds ($P = 0.51$; Figure 2b).

Plumage water retention did not differ among treatments ($F_{2,74} = 2.47$, $P = 0.09$; Figure 5). Water retention was higher in birds in lower body condition than in better body condition ($F_{1,76} = 4.35$, $P = 0.040$).

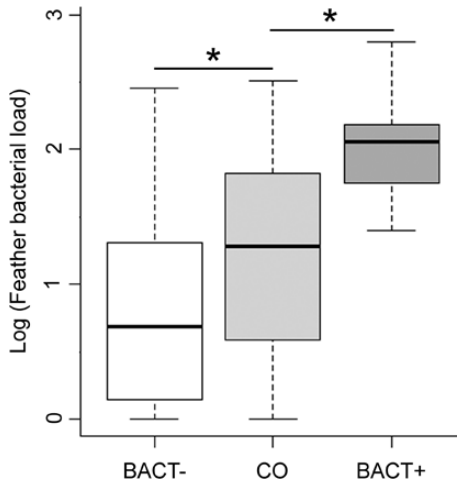


Figure 3
Boxplot of feather bacterial load in BACT−, CO, and BACT+ pigeons. *$P < 0.05$.

Brightness of iridescent neck feathers differed among treatments ($F_{2,71} = 4.36$, $P = 0.024$; Figure 1). BACT− birds had lower brightness than BACT+ birds ($P = 0.029$; Figure 1), while CO birds had intermediate brightness but not significantly different from BACT− and BACT+ birds (CO vs. BACT−: $P = 0.41$, CO vs. BACT+: $P = 0.37$; Figure 1). Interestingly, we found a significant interaction between sex and color morph on brightness ($F_{1,71} = 13.10$, $P < 0.0001$). In males, paler pigeons had lower brightness than darker pigeons ($F_{1,34} = 5.66$, $P = 0.023$), while in females, paler pigeons had higher brightness ($F_{1,39} = 5.94$, $P = 0.019$). The Q4 quantum catch (the long-wavelength-sensitive photoreceptor response) differed between neck feathers of males and females (mean ± SE: 0.24 ± 0.00 and 0.26 ± 0.01; $F_{1,75} = 6.05$, $P = 0.016$), and the Q1 quantum catch (the violet-sensitive photoreceptor response) tended to differ between neck feathers of males and females ($F_{1,75} = 3.15$, $P = 0.08$).

DISCUSSION

As predicted, pigeons with lower bacterial load on feathers had higher quality plumage. Avian feathers host keratinolytic bacteria (Burtt and Ichida 1999; Whitaker et al. 2005), which have the ability to degrade feathers in vitro (Burtt and Ichida 1999; Cristol et al. 2005; Shawkey et al. 2007; Ruiz-De-Castaneda et al. 2012). However, whether they degrade feathers in vivo has remained unknown. So far, the only experimental study on live birds has tested only one strain of keratinolytic bacteria and has shown no effect of the bacteria on feather condition (Cristol et al. 2005). Our study provides the first in vivo experimental evidence of a negative effect of plumage bacteria on feather condition, hence validating previous in vitro experiments.

As predicted, pigeons with lower feather bacterial load had a lower quantity of secretion within the preen gland and preened their feathers less often. Although pigeons have small preen gland compared to other birds (Montalti et al. 2005), our study seems to validate its functionality in this species, as found in a previous experiment showing positive effects of preen oil on feather condition in pigeons (Moyer et al. 2003). As suggested in previous studies

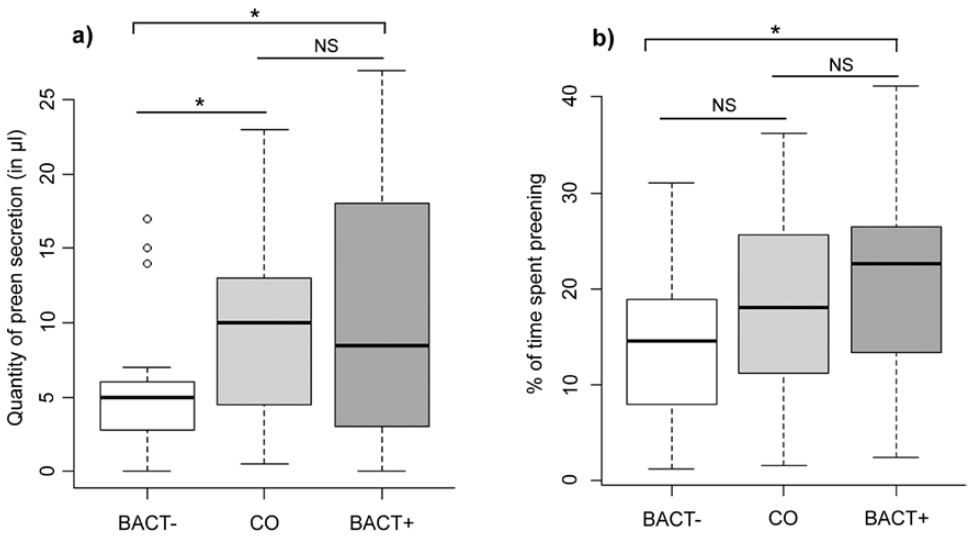


Figure 4
(a) Boxplot of quantity of preen secretion within the gland and (b) Boxplot of percentage of time spent preening in BACT−, CO, and BACT+ pigeons. *$P < 0.05$, NS: $P > 0.05$.

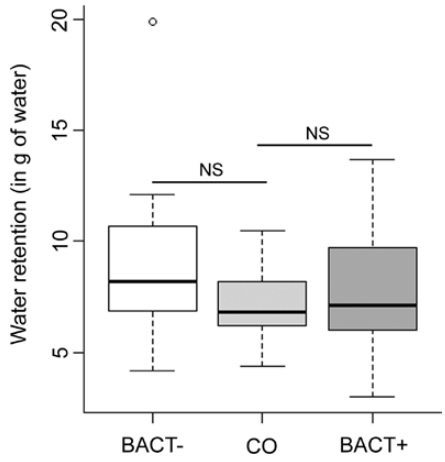


Figure 5
Boxplot of water retention (grams of water retained in plumage) in BACT−, CO, and BACT+ pigeons. NS: $P > 0.05$.

(Reneerkens et al. 2008), preen oil may reduce bacterial load by acting as a physical barrier that prevents bacteria to reach feathers. Nonexclusively preen oil may contain antibacterial substances, either lipids (Bandyopadhyay and Bhattacharyya 1996; Jacob et al. 1997) or peptides produced by bacteria hosted in the gland (Martín-Vivaldi et al. 2010). Our result shows that the production of preen oil may be induced by the presence of environmental pressures (i.e., high plumage bacterial load). According to the theory of inducible defense (Harvell 1990), if preen secretion and preening behavior are inducible, they may be costly. This cost may arise from the physiological costs of producing preen secretion or large preen gland or from the fact that preening, being time-consuming, may reduce time devoted to other behavior such as feeding or sleeping (Christe et al. 1996). In tawny owls *Strix aluco*, birds stimulated to produce an immune response have smaller preen gland (Piault et al. 2008), and in apapanes *Himatione sanguinea*, birds infected by *plasmodium* preen less frequently (Yorinks and Atkinson 2000). Whatever the mechanism behind the cost, the consequence of this induced response may have important consequences in life history strategies. Birds with low bacterial load on feathers may save energy from this costly response for other life history traits. However, the cost of this response needs to be tested experimentally by distinguishing the negative effects of bacterial load from the induced response. It is the next methodological challenge to address this question for future studies.

Using an avian visual model, we found that birds with lower bacteria load on plumage had less bright neck feathers. In eastern bluebirds, feather bacteria load is positively related to brightness of structural UV-blue plumage color and in vitro experiments has confirmed that keratinolytic bacteria degrade fathers and brighten them. Bacterial degradation of the light-absorbing cortex of bluebirds' feathers may cause greater reflection of light and hence higher brightness (Shawkey et al. 2007). In contrast to bluebirds, structural colors of pigeons originate from the thin-film interference of the top keratin cortex layer, while brightness partly originates from the medullary layer (Yin et al. 2006). It suggests that changes in brightness coupled with lack of change in chromatic color are probably not associated with bacterial degradation of the barbule cortex layer. Further studies should examine whether changes in brightness in pigeons' feathers are due to modifications of feather structure. Alternatively, decreased preen secretion onto the plumage of birds with lower bacterial load may have directly decreased feather brightness. However, evidence in mallards and tawny owls suggest that preen oil decreases, rather than increases, integument brightness (Delhey et al. 2008; Piault et al. 2008). Although the importance of neck feather coloration in signaling in pigeons is unknown, several observations suggest that it may play a role in sexual selection. During agonistic and courtship behavior, males display their neck feathers at conspecifics (Johnston 1992). Furthermore, our results show that males and females differ in the chromatic coloration of neck feathers, and that this difference occurs mainly in the long wavelength range. To humans, females seem also less iridescent than males (Johnston 1992), although confirmation of this observation would require measurements of iridescence properties and quantity of iridescent feathers. Feather bacteria may therefore play an important role in the evolution of the signaling function of neck feather color in pigeons. Neck feather color might reveal bacterial damage and therefore be scrutinized by conspecifics during mate choice or competition.

Feather bacterial load did not seem to affect water repellency efficiency of the plumage. Water repellency efficiency of the plumage depends on feather structure (Rijke 1970), but also on the quantity of preen secretions (van Rhijn 1977), which contain hydrophobic compounds (Montalti et al. 2005; Leclaire et al. 2011; Campagna et al. 2012). In our study, plumage quality is negatively related to preen secretion quantity and preening. If both factors affect water retention in pigeons, they may compensate each other, which result in a lack of differences between treated and control birds. Studies including experimental increase of preen secretion on feathers of varying condition, and hydrophobicity measurements using, for instance, contact angle between water droplet and feathers (see Eliason and Shawkey 2011) would be needed to test this hypothesis.

To experimentally decrease bacterial load on feathers, birds were sprayed twice a week with a chlorhexidine solution. Chlorhexidine has not been shown to have adverse effects, and it is not irritating to the skin when concentration is lower than 2%. However, it might remove natural oils and emollients and therefore frequent washing may result in skin dryness. Birds sprayed with chlorhexidine would therefore have been expected to invest more in preening and to have degraded feathers. This alternative explanation is however unlikely as our results show the opposite pattern, suggesting that the observed effects are not due to chlorhexidine per se, but on its effect in decreasing bacteria load.

In conclusion, our study demonstrates, for the first time in vivo, that feather bacteria degrade feathers and alter iridescent coloration, thus potentially affecting visual signals involved in sexual or social competition. It further suggests that birds may invest in preening—a likely costly defensive traits—depending on the load of feather bacteria. Feral pigeons live in highly urbanized habitats which are known to harbor high bacterial densities (Shaffer and Lighthart 1997). Further studies should now evaluate whether wild urban pigeons have elaborated strategies, such as enlarged preen gland, to prevent feather degradation. Another strategy may be the elaboration of a darker plumage, as melanin-colored feathers are more resistant to bacterial degradation than unmelanized feathers (Ruiz-De-Castaneda et al. 2012). Urban feral pigeons display a darker melanin-based coloration than rural individuals (Johnston and Janiga 1995), but whether it reflects adaptation to habitat with high bacterial load needs now to be studied.

FUNDING

This work was financed by a grant from the local government (Ile-de-France: Sustainable Development Network R2DS, No. 2012-11).

We thank S. Perret, F. Ruiz, L. Forge, R. Guttierez, A. Hammouda, M. Martin, and S. Jacob for their help at various stages of the experiment. This study was carried out in strict accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (revised Appendix A). All experiments and captures were approved by local authorities and the "Direction Départementale des Services Vétérinaires de Seine-et-Marne" (permit No. 77-05).

Handling editor: Marc Thery

REFERENCES

Archie EA, Hollister-Smith JA, Poole JH, Lee PC, Moss CJ, Maldonado JE, Fleischer RC, Alberts SC. 2007. Behavioural inbreeding avoidance in wild African elephants. Mol Ecol. 16:4138–4148.

Bandyopadhyay A, Bhattacharyya SP. 1996. Influence of fowl uropygial gland and its secretory lipid components on growth of skin surface bacteria of fowl. Indian J Exp Biol. 34:48–52.

Bowmaker JK, Heath LA, Wilkie SE, Hunt DM. 1997. Visual pigments and oil droplets from six classes of photoreceptor in the retinas of birds. Vision Res. 37:2183–2194.

Burtt EH, Ichida JM. 1999. Occurrence of feather-degrading bacilli in the plumage of birds. Auk. 116:364–372.

Campagna S, Mardon J, Celerier A, Bonadonna F. 2012. Potential semiochemical molecules from birds: a practical and comprehensive compilation of the last 20 years studies. Chem Senses. 37:3–25.

Christe P, Richner H, Oppliger A. 1996. Of great tits and fleas: sleep baby sleep. Anim Behav. 52:1087–1092.

Clayton DH. 1999. Feather-busting bacteria. The Auk. 116:302–304.

Cogen AL, Nizet V, Gallo RL. 2008. Skin microbiota: a source of disease or defence? Br J Dermatol. 158:442–455.

Cristol DA, Armstrong JL, Whitaker JM, Forsyth MH, Sodhi N. 2005. Feather-degrading bacteria do not affect feathers on captive birds. The Auk. 122:222–230.

Czirják GA, Pap PL, Vágási CI, Giraudeau M, Muresan C, Mirleau P, Heeb P. 2013. Preen gland removal increases plumage bacterial load but not that of feather-degrading bacteria. Naturwissenschaften. 100:145–151.

Delhey K, Peters A, Biedermann PH, Kempenaers B. 2008. Optical properties of the uropygial gland secretion: no evidence for UV cosmetics in birds. Naturwissenschaften. 95:939–946.

Eliason CM, Shawkey MD. 2011. Decreased hydrophobicity of iridescent feathers: a potential cost of shiny plumage. J Exp Biol. 214:2157–2163.

Endler JA, Mielke PW. 2005. Comparing entire colour patterns as birds see them. Biol J Linn Soc. 86:405–431.

Ezenwa VO, Gerardo NM, Inouye DW, Medina M, Xavier JB. 2012. Microbiology. Animal behavior and the microbiome. Science. 338:198–199.

Giraudeau M, Duval C, Guillon N, Bretagnolle V, Gutierrez C, Heeb P. 2010. Effects of access to preen gland secretions on mallard plumage. Naturwissenschaften. 97:577–581.

Gunderson AR. 2008. Feather-degrading bacteria: a new frontier in avian and host-parasite research? Auk. 125:972–979.

Hagelin JC, Jones IL. 2007. Bird odors and other chemical substances: a defense mechanism or overlooked mode of intraspecific communication? Auk. 124:741–761.

Hart BL. 1988. Biological basis of the behavior of sick animals. Neurosci Biobehav Rev. 12:123–137.

Hart NS, Vorobyev M. 2005. Modelling oil droplet absorption spectra and spectral sensitivities of bird cone photoreceptors. J Comp Physiol A Neuroethol Sens Neural Behav Physiol. 191:381–392.

Harvell CD. 1990. The ecology and evolution of inducible defenses. Q Rev Biol. 65:323–340.

Jacob J, Eigener U, Hoppe U. 1997. The structure of preen gland waxes from pelecaniform birds containing 3, 7-dimethyloctan-1-ol: an active ingredient against dermatophytes. Z Naturfosch. 52:114–123.

Jacob J, Ziswiler V. 1982. The uropygial gland. In: Farner DS, King JR, Parkes KC, editors. Avian biology. New York: Academic Press. p. 199–324.

Jacquin L, Haussy C, Bertin C, Laroucau K, Gasparini J. 2013. Darker female pigeons transmit more specific antibodies to their eggs than do paler ones. Biol J Linnean Soc. 108:647–657.

Jacquin L, Lenouvel P, Haussy C, Ducatez S, Gasparini J. 2011. Melanin-based coloration is related to parasite intensity and cellular immune response in an urban free living bird: the feral pigeon *Columba livia*. J Avian Biol. 42:11–15.

Johnston RF. 1992. Rock dove: *Columba livia*. In: Poole A, Stettenheim P, Gill F, editors. The birds of North America. Vol 13. Philadelphia, PA: Academy of Natural Sciences and Washington, DC: the American Ornithologists' Union.

Johnston RF, Janiga M. 1995. Feral pigeons. New York: Oxford University Press.

Leclaire S, Merkling T, Raynaud C, Giacinti G, Bessière JM, Hatch SA, Danchin E. 2011. An individual and a sex odor signature in kittiwakes?: study of the semiochemical composition of preen secretion and preen down feathers. Naturwissenschaften. 98:615–624.

Lind O, Mitkus M, Olsson P, Kelber A. 2014. Ultraviolet vision in birds: the importance of transparent eye media. Proc Biol Sci. 281:20132209.

Maia R, Eliason CM, Bitton P-P, Doucet SM, Shawkey MD. 2013. pavo: an R package for the analysis, visualization and organization of spectral data. Methods Ecol Evol. 4:906–913.

Martín-Vivaldi M, Peña A, Peralta-Sánchez JM, Sánchez L, Ananou S, Ruiz-Rodríguez M, Soler JJ. 2010. Antimicrobial chemicals in

hoopoe preen secretions are produced by symbiotic bacteria. Proc Biol Sci. 277:123–130.
McGraw KJ. 2004. Multiple UV reflectance peaks in the iridescent neck feathers of pigeons. Naturwissenschaften. 91:125–129.
Møller AP, Czirjak GA, Heeb P. 2009. Feather micro-organisms and uropygial antimicrobial defences in a colonial passerine bird. Funct Ecol. 23:1097–1102.
Montalti D, Gutiérrez AM, Reboredo G, Salibián A. 2005. The chemical composition of the uropygial gland secretion of rock dove *Columba livia*. Comp Biochem Physiol, Part A Mol Integr Physiol. 140:275–279.
Moyer BR, Rock AN, Clayton DH. 2003. Experimental test of the importance of preen oil in rock doves (*Columba livia*). Auk. 120:490–496.
Piault R, Gasparini J, Bize P, Paulet M, McGraw KJ, Roulin A. 2008. Experimental support for the makeup hypothesis in nestling tawny owls (*Strix aluco*). Behav Ecol. 19:703–709.
R Development Core Team. 2014. R: a language and environment for statistical computing. Vienna (Austria): R Foundation for Statistical Computing.
Reneerkens J, Versteegh MA, Schneider AM, Piersma T, Burtt EH. 2008. Seasonally changing preen-wax composition: red knots' (*Calidris canutus*) flexible defense against feather-degrading bacteria? The Auk. 125:285–290.
Rijke AM. 1970. Wettability and phylogenetic development of feather structure in water birds. J Exp Biol. 52:469–479.
Ruiz-De-Castaneda R, Burtt EH, Jr, Gonzalez-Braojos S, Moreno J. 2012. Bacterial degradability of an intrafeather unmelanized ornament: a role for feather-degrading bacteria in sexual selection? Biol J Linnean Soc. 105:409–419.
Scott DW. 2001. Bacterial skin diseases. In: Scott DW, editor. Color Atlas of Farm Animal Dermatology. Oxford, UK: Blackwell Publishing Ltd. p. 151–155.
Shaffer BT, Lighthart B. 1997. Survey of culturable airborne bacteria at four diverse locations in Oregon: Urban, Rural, Forest, and Coastal. Microb Ecol. 34:167–177.
Sharon G, Segal D, Ringo JM, Hefetz A, Zilber-Rosenberg I, Rosenberg E. 2010. Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. Proc Natl Acad Sci USA. 107:20051–20056.
Shawkey MD, Hill GE. 2004. Feathers at a fine scale. The Auk. 121:652–655.
Shawkey MD, Pillai SR, Hill GE. 2003. Chemical warfare? Effects of uropygial oil on feather-degrading bacteria. J Avian Biol. 34:345–349.
Shawkey MD, Pillai SR, Hill GE, Siefferman LM, Roberts SR. 2007. Bacteria as an agent for change in structural plumage color: correlational and experimental evidence. Am Nat. 169(suppl. 1):S112–S121.
Swaddle JP, Witter MS, Cuthill IC, Budden A, McCowen P. 1996. Plumage condition affects flight performance in common starlings: implications for developmental homeostasis, abrasion and moult. J Avian Biol. 27:103–111.
Tannock GW. 1995. Normal microflora: an introduction to microbes inhabiting the human body. London, UK: Chapman & Hall.
Tollrian R, Harvell CD. 1999. The ecology and evolution of inducible defenses. Princeton, NJ: Princeton University Press.
van Rhijn JG. 1977. Processes in feathers caused by bathing in water. Ardea. 65:126–147.
Whitaker JM, Cristol DA, Forsyth MH. 2005. Prevalence and genetic diversity of *Bacillus licheniformis* in avian plumage. J Field Ornithol. 76:264–270.
Wolak ME, Fairbairn DJ, Paulsen YR. 2012. Guidelines for estimating repeatability. Methods Ecol Evol. 3:129–137.
Yin H, Shi L, Sha J, Li Y, Qin Y, Dong B, Meyer S, Liu X, Zhao L, Zi J. 2006. Iridescence in the neck feathers of domestic pigeons. Phys Rev E Stat Nonlin Soft Matter Phys. 74:051916.
Yorinks N, Atkinson CT. 2000. Effects of malaria on activity budgets of experimentally infected juvenile Apapane (*Himatione sanguinea*). Auk. 117:731–738.
Yoshioka S, Nakamura E, Kinoshita S. 2007. Origin of two-color iridescence in rock dove's feather. J Phys Soc Jpn. 76:013801.

Métaux traces : réponses écophysiologiques et rôle dans le maintien du polymorphisme de coloration mélanique du plumage chez le pigeon biset

Résumé :

Les métaux traces comme le plomb, le zinc sont essentiellement émis par les activités anthropiques et se retrouvent de ce fait à des concentrations beaucoup plus élevées en milieux urbains qu'en milieux ruraux. Durant ma thèse, j'ai tout d'abord testé les effets écotoxicologiques d'une exposition chronique au plomb et/ou au zinc, deux métaux particulièrement abondants en milieu urbain, chez le pigeon biset (*Columba livia*). J'ai ainsi pu montrer des effets nocifs du plomb, et bénéfiques du zinc sur l'immunité, le maintien de la corpulence et plusieurs paramètres de la reproduction. Du fait de la variabilité des réponses écophysiologiques des individus, les métaux traces sont susceptibles d'exercer de nouvelles pressions de sélection sur les populations urbaines et favoriser les individus capables de se détoxifier ou de tolérer de fortes concentrations en métaux. Au cours de ma thèse, j'ai mis en évidence le rôle de la mélanine dans la fixation du zinc et du plomb au niveau des plumes. Par ailleurs la coloration mélanique du plumage semblent moduler les effets du plomb et du zinc sur certains paramètres physiologiques, et les juvéniles au plumage davantage mélanique survivent mieux dans un environnement pollué en plomb. Quels que soient les mécanismes sous-jacents (i.e. rôle détoxifiant de la mélanine ou effets pléiotropes associés à sa synthèse), mes résultats soulignent l'avantage sélectif potentiel de la mélanisation du plumage dans un environnement pollué en métaux traces, dont notamment le milieu urbain. Cette étude apporte des réponses essentielles sur l'impact écologique de l'urbanisation et les mécanismes permettant le maintien du polymorphisme de coloration mélanique du plumage, et plus largement des phanères.

Mots clés : métaux traces, écotoxicologie, écophysiologie, écologie urbaine, coloration mélanique

Trace metals: ecophysiological responses and their influence on melanin-based plumage colouration polymorphism maintenance in feral pigeons

Abstract:

Trace metals, such as lead and zinc are mainly emitted by human activities, explaining their high concentrations in urban areas in comparison with rural environments. During my PhD, I first investigated the ecotoxicological effects of a chronic exposure to lead and/or zinc, two abundant metals in urban areas, in feral pigeons (*Columba livia*). I stressed deleterious effects of lead, while beneficial effects of zinc on immunity, body mass index maintenance and several parameters of reproduction. Because sensitivity to trace metals differs between individuals, trace metals may exert new selective pressures on urban populations and favour individuals with higher detoxification capacities and that are more tolerant to elevated environmental trace metals concentrations. My work puts ahead the role of melanin in the storage of zinc and lead in the feathers. Moreover, melanin-based plumage colouration seems to modulate the effects of lead and zinc on some of the physiological parameters measured and darker juveniles were more prone to survive than paler ones when exposed to lead. Whatever the underlying mechanism (i.e. the detoxification role of melanin or the pleiotropic effects associated with its synthesis), my results suggest a selective advantage of plumage melanism in environments polluted with trace metals, such as urban areas. This study brings key answers on the ecological impact of urbanization and on the mechanisms explaining melanin-based plumage colouration polymorphism maintenance.

Keywords: trace metals, ecotoxicology, ecophysiology, urban ecology, melanin-based colouration